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7	Appendix B
8	Protocol for the LUMI-CELL® Estrogen Receptor (ER) Transcriptional Activation Test

Method for the Detection of ER Agonists

06 April 2007

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06 April 2007

AGONIST PROTOCOL LUMI-CELL® ESTROGEN RECEPTOR TRANSCRIPTIONAL ACTIVATION TEST METHOD FOR IDENTIFYING ESTROGEN AGONISTS AND ANTAGONISTS National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative **Toxicological Methods (NICEATM) Developed by: Xenobiotic Detection Systems, Inc.** 1601 E. Geer St., Suite S Durham, NC 27704

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155	13 mm test tube	13 x 100 mm glass test tubes
156	Absolute EC ₅₀ value	Concentration of a substance that increases the measured
157		activity in an agonist assay to 50% of the maximum activity
158		induced by the reference substance
159	CASRN	Chemical Abstracts Service Registry Number
160	DMEM	Dulbecco's Modification of Eagle's Medium
161	DMSO	Dimethyl Sulfoxide
162	DMSO control	1% v/v dilution of DMSO in tissue culture media used as a
163		vehicle control
164	E2	17β-estradiol
165	E2 reference standard	10 Point Serial Dilution of 17β-estradiol reference standard
166		for the LUMI-CELL® ER agonist assay
167	EC ₅₀	Concentration of test chemical causing half of maximal
168		response
169	ER	Estrogen Receptor
170	Estrogen-free DMEM	DMEM (phenol red free) supplemented with 1%
171		Penicillin/Streptomycin, 2% L-Glutamine, and 5%
172		Charcoal-dextran treated FBS
173	FBS	Fetal Bovine Serum
174	G418	Gentamycin
175	GLP	Good Laboratory Practice
176	Methoxychlor	<i>p,p'</i> -Methoxychlor
177	Methoxychlor control	3.13 µg/mL Methoxychlor Positive Control for the LUMI-
178		CELL® ER Agonist Assay
179	PBS	Phosphate-buffered Saline
180	Relative EC ₅₀ value	Concentration that produces a half-maximal response as
181		calculated using the four parameter Hill function.
182	RLU	Relative Light Units
183	RPMI	RPMI 1640 growth medium
184	SMT	Study Management Team
185	SOP	Standard Operating Procedure
186	TA	Transcriptional Activation
187	T25	25 cm ² tissue culture flask
188	T75	75 cm ² tissue culture flask
189	T150	150 cm ² tissue culture flask
190		

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- **1.0 PURPOSE**
- 243 This protocol is designed to evaluate coded test substances for potential estrogen receptor (ER)
- agonist activity using the LUMI-CELL® ER assay.
- **245 2.0 SPONSOR**
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- Toxicological Methods (NICEATM), P.O. Box 12233 Research Triangle Park, NC 27709
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301	Chemistry Resources Group Leader
302	National Institute of Environmental Health Sciences
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304	Research Triangle Park, NC 27709
305	Phone: 919-541-3473
306	

30/	3.0	DEFINITIONS
308 309		• Dosing Solution: The test substance, control substance, or reference standard solution, which is to be placed into the tissue culture wells for experimentation.
310 311		• Raw Data: Raw data includes information that has been collected but not formatted or analyzed, and consists of the following:
312		 Data recorded in the Study Notebook
313		 Computer printout of initial luminometer data
314		 Other data collected as part of GLP compliance, e.g.:
315		Equipment logs and calibration records
316		 Test substance and tissue culture media preparation logs
317		• • •
		Cryogome needer inventory rogs
318 319		• Soluble: Test substance exists in a clear solution without visible cloudiness or precipitate.
320		• Study Notebook: The study notebook contains recordings of all activities related
321		to the conduct of the LUMI-CELL® ER TA agonist assay.
322		• Test Substances: Substances supplied to the testing laboratories that are coded
323		and distributed such that only the Project Officer, Study Management Team
324		(SMT), and the Substance Inventory and Distribution Management have
325		knowledge of their true identity. The test substances will be purchased, aliquoted,
326 327		coded, and distributed by the Supplier under the guidance of the NIEHS/NTP Project Officer and the SMT.
328	4.0	TESTING FACILITY AND KEY PERSONNEL
329	4.1	Testing Facility
330	Xenob	iotic Detection Systems, Inc. (XDS), 1601 E. Geer St., Suite S, Durham, NC 27704
331	4.2	Key Personnel
332		• Study Director: John Gordon, Ph.D.
333		• Laboratory Technician(s): Cynthia Matherly
334		Scientific Advisor: Mike Denison, Ph.D.
335		Quality Assurance Director: Andrew Chu
336		Safety Manager: George Clark, Ph.D.

337 338		• Facility Management: George Clark, Ph.D., Robert Clark, Tina Ginter, Andrew Chu
339		Laboratory Director: Andrew Chu
340	5.0	IDENTIFICATION OF TEST AND CONTROL SUBSTANCES
341	5.1	Test Substances
342 343		ostances are coded and will be provided to participating laboratories by the Substance ry and Distribution Management team.
344	5.2	Controls
345	Control	s for the ER agonist protocol are as follows:
346 347		control (dimethyl sulfoxide [DMSO]): 1% (v/v) DMSO (CASRN 67-68-5) diluted in ulture media.
348 349 350	duplicat	ce standard (17β-estradiol [E2]): Three concentrations of E2 (CASRN 50-28-2) in the for range finder testing and a serial dilution consisting of 10 concentrations of E2 in the for comprehensive testing
351 352		e control (p,p'-Methoxychlor [methoxychlor]): Methoxychlor (CASRN 72-43-5), 3.13 in tissue culture media, used as a weak positive control.
353	6.0	OVERVIEW OF GENERAL PROCEDURES FOR AGONIST TESTING
354 355 356	glasswa	erimental procedures are to be carried out under aseptic conditions and all solutions, are, plastic ware, pipettes, etc., shall be sterile. All methods and procedures shall be ented in the study notebook.
357 358 359	⁴ , 2.00 x	range finder testing is conducted on 96-well plates using three concentrations (1.00 x 10 x 10^{-6} , 1.00 x 10^{-7} µg/mL) of E2 in duplicate as the reference standard and six replicate or the DMSO control. Range finder testing uses all wells of the 96-well plate.
360 361 362 363 364	as the re replicate wells or	chensive testing is conducted on 96-well plates using 10 concentrations of E2 in duplicate reference standard (Table 6-1). Four replicate wells for the DMSO control and three wells for the methoxychlor control are included on each plate. To avoid edging effects the perimeter of the plate are not used for experiments. These wells should contain alture media only.

¹ Edging effects are variations in response seen in the outermost wells in a cell culture plate. These variations are believed to be due to variations in temperature, evaporation, etc., that may occur in these wells that would ultimately affect cellular growth and health (ICCVAM, 2001).

Table 6-1 Concentrations of E2 Reference Standard Used in Comprehensive Testing

E2 Concentrations ¹					
1.00 x 10 ⁻⁴	6.25 x 10 ⁻⁶	1.95 x 10 ⁻⁷			
5.00 x 10 ⁻⁵	3.13 x 10 ⁻⁶	9.78 x 10 ⁻⁸			
2.50 x 10 ⁻⁵	1.56 x 10 ⁻⁶				
1.25 x 10 ⁻⁵	7.83 x 10 ⁻⁷				

¹Concentrations are presented in µg/mL.

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Visual observations for cell viability are conducted for all experimental plates just prior to

LUMI-CELL® ER evaluation. CellTiter-Glo® based cell viability testing (when used) is

conducted concurrently in parallel plates, as outlined in **Section 11.2**.

370 Luminescence data, measured in relative light units (RLUs), is corrected for background

luminescence by subtracting the mean RLU value of the vehicle control (DMSO) wells from the

RLU measurements for each of the other wells of the 96-well plate. Data is then transferred into

Excel® data management spreadsheets and GraphPad PRISM® 4.0 statistical software, graphed,

and evaluated as follows:

- A response is considered positive for agonist activity when the average adjusted RLU for a given concentration is greater than the mean RLU value plus three times the standard deviation for the vehicle control.
- Any response below this threshold is considered negative for agonist activity.
- For substances that are positive at one or more concentrations, the concentration that causes a
- half-maximal response (EC_{50}) is calculated using a Hill function analysis. The Hill function is a
- four-parameter logistic mathematical model relating the substance concentration to the response
- 382 (typically following a sigmoidal curve) using the equation below:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(logEC50-X)HillSlope}}$$

384 where Y = response (i.e., relative light units); X = the logarithm of concentration; Bottom = the

minimum response; Top = the maximum response; $\log EC_{50}$ = the logarithm of X as the response

midway between Top and Bottom; and HillSlope describes the steepness of the curve. The model

calculates the best fit for the Top, Bottom, HillSlope, and EC₅₀ parameters. See Section 11.6.5

388 for more details.

389 Acceptance or rejection of a test is based on evaluation of reference standard and control results

from each experiment conducted on a 96-well plate. Results for these controls are compared to

391 historical results compiled in the historical database, as seen in **Section 14.0**.

392 **6.1** Range Finder Testing

- 393 Agonist range finding for coded substances consists of a six point, logarithmic serial dilution
- 394 using duplicate wells per concentration. Concentrations for comprehensive testing are selected
- based on the response observed in range finder testing. If necessary, a second range finder test
- can be conducted to clarify the optimal concentration range to test (see **Section 12.0**).

397 **6.2** Comprehensive Testing

- 398 Comprehensive agonist testing for coded substances consists of 11 point, double serial dilutions,
- with each concentration tested in triplicate wells of the 96-well plate. Three separate experiments
- are conducted for comprehensive testing on three separate days, except during Phases III and IV
- of the validation effort, in which comprehensive testing experiments are conducted once (see
- 402 **Section 13.0**).

409

403 7.0 MATERIALS FOR LUMI-CELL® ER AGONIST TESTING

- This section provides the materials needed to conduct LUMI-CELL® ER testing, with associated
- 405 brand names/vendors² in brackets.

406 **7.1 BG1Luc4E2 Cells:**

- Human ovarian cancer cell line stably transfected with a plasmid containing an estrogen response
- 408 element pGudLuc7.0 (**Figure 7-1**) [XDS].

⁴⁰⁹

²Brand names and vendors should not be considered an endorsement by the U.S. Government or any member of the U.S. Government; such information is provided as examples.

409 Figure 7-1 pGudLuc7.ERE Plasmid. 410 411 7.2 **Technical Equipment:** 412 All technical equipment may be obtained from Fisher Scientific International, Inc. (Liberty Lane 413 Hampton, NH, USA 03842). Equivalent technical equipment from another commercial source 414 can be used. 415 Analytical balance (Cat. No. 01-910-320) 416 Berthold Orion 1 Microplate Luminometer [Berthold CatNo.: Orion 1 MPL3] or 417 equivalent and dedicated computer 418 Biological safety hood, class II, and stand (Cat. No. 16-108-99) 419 Centrifuge (low speed, tabletop with swinging bucket rotor) (Cat. No. 04-978-50 420 centrifuge, and 05-103B rotor) 421 Combustion test kit (CO₂ monitoring) (Cat. No. 10-884-1) 422 Drummond diaphragm pipetter (Cat. No. 13-681-15) 423 Freezers, -20°C (Cat. No. 13-986-150), and -70°C (Cat. No. 13-990-86) 424 Hand tally counter (Cat. No. 07905-6) 425 Hemocytometer, cell counter (Cat. No. 02-671-5) 426 Light microscope, inverted (Cat. No. 12-561-INV) 427 Light microscope, upright (Cat. No. 12-561-3M) 428 Liquid nitrogen flask (Cat. No. 11-675-92) 429 Micropipetter, repeating (Cat. No. 21-380-9) 430 Pipetters, air displacement, single channel (0.5 –10µl (Cat. No. 21-377-191), 2 – 431 20 μl (Cat. No. 21-377-287), 20 – 200 μl (Cat. No. 21-377-298), 200 - 1000 μl 432 (Cat. No. 21-377-195))

433	•	Refrigerator/freezer (Cat. No. 13-986-106A)
434	•	Shaker for 96-well plates (Cat. No. 14-271-9)
435	•	Sodium hydroxide (Cat. No. 5318-500)
436	•	Sonicating water bath (Cat. No. 15-335-30)
437	•	Tissue culture incubator with CO ₂ and temperature control (Cat. No. 11-689-4)
438	•	• Vacuum pump with liquid trap (side arm Erlenmeyer) (Cat. No. 01-092-29)
439	•	Vortex mixer (Cat. No. 12-814)
440 441	Equipment SOPs.	should be maintained and calibrated as per GLP guidelines and individual laboratory
442	7.3	Reference Standard, Controls, and Tissue Culture Supplies
443 444 445	expiration of	culture reagents must be labeled to indicate source, identity, storage conditions and dates. Tissue culture solutions must be labeled to indicate concentration, stability own), and preparation and expiration dates.
446 447	•	tissue culture media and sera from another commercial source can be used, but must ed as described in Section 15.0 to determine suitability for use in this test method.
448 449		ing are the necessary tissue culture reagents and possible commercial sources (in ased on their use in the pre-validation studies:
450	•	• 17 β-estradiol (CAS RN: 50-28-2) [Sigma-Aldrich, Cat. No. E8875]
451	•	• CellTiter-Glo® Luminescent Cell Viability Assay [Promega Cat. No. G7572]
452	•	Cryovial, 2 mL (Corning Costar) [Fisher Scientific Cat. No. 03-374-21]
453	•	Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 10009186R38]
454 455	•	Culture tube, 50 mL conical (Corning Costar) [Fisher Scientific Cat. No. 05-526C]
456	•	DMSO, U.S.P. analytical grade. [Sigma-Aldrich, Cat. No. 34869-100ML]
457 458 459	•	Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L glucose, with sodium pyruvate, without phenol red or L-glutamine [Mediatech/Cellgro, Cat. No. 17-205-CV]
460		Fetal Bovine Serum [Mediatech/Cellgro Cat. No. MT 35-010-CV]
461		Fetal Bovine Serum, charcoal/dextran treated, triple 0.1 µm sterile filtered
462		[Hyclone, Cat. No. SH30068.03]
463	•	Gentamycin Sulfate (G418), 50 mg/mL [Mediatech/Cellgro Cat. No. 30-234-CR]
464	•	L-glutamine, 29.2 mg/mL [Cellgro, Cat. No. 25005-CI]

465 Luciferase Assay System (10-Pack) [Promega Cat. No. E1501] 466 Lysis Solution 5X [Promega, Cat. No. E1531] 467 Methoxychlor (CAS RN: 72-43-5) [Sigma-Aldrich, Cat. No. 49054] 468 Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 µg/mL streptomycin [Cellgro, Cat. No. 30-001-CI]. 469 470 Phosphate buffered saline (PBS, 1X) without calcium and magnesium [Cellgro, 471 Cat. No. 21-040-CV] 472 Pipettes, serological: 2.0 mL [Sigma-Aldrich, Cat. No. P1736], 5.0 mL [Sigma-473 Aldrich, Cat. No. P1986], 25 mL [Sigma-Aldrich, Cat. No. P2486] 474 RPMI 1640 medium, containing L-glutamine [Mediatech, Cat. No. 10-040-CV] Tissue culture flasks (Corning-Costar): 25 cm² (T25) [Fisher Cat. No. 10-126-28]; 475 75 cm² (T75) [Fisher Cat. No. 10-126-37]; and 150 cm² (T150) [Fisher Cat. No. 476 10-126-34] 477 Tissue culture plates (Corning-Costar): 96-well [Thomas Scientific Cat. No. 478 479 6916A05] 480 Trypsin (10X), 2.5% in Hank's balanced salt solution (HBSS), without calcium 481 and magnesium, without phenol red [Cellgro, Cat. No. 25-054-CI]. 482 All reagent lot numbers and expiration dates must be recorded in the study notebook. 483 8.0 PREPARATION OF TISSUE CULTURE MEDIA AND SOLUTIONS 484 All tissue culture media and media supplements must be quality tested before use in experiment 485 (see Section 15.0). 486 8.1 RPMI 1640 Growth Medium (RPMI) 487 RPMI 1640 is supplemented with 0.9% Pen-Strep and 8.0% FBS to make RPMI growth medium 488 (RPMI). 489 Procedure for one 549 mL bottle: Remove FBS from -70°C freezer, and Pen-Strep from -20°C freezer and allow to 490 491 equilibrate to room temperature. 492 2. Add 44 mL of FBS and 5 mL Pen-Strep to the bottle of RPMI 1640.

C-255

Store at 2-8°C for no longer than six months or until the shortest expiration date of any media

3. Label RPMI bottle as indicated in **Section 7.3**

493

494

495

component.

496 **8.2** Estrogen-Free DMEM Medium

- DMEM is supplemented to contain 4.5% charcoal/dextran treated FBS, 1.9% L-glutamine, 0.9%
- 498 Pen-Strep.
- 499 Procedure for one 539 mL bottle:
- 1. Remove charcoal/dextran treated FBS from -70°C freezer, and L-glutamine and Pen-Strep from -20°C freezer and allow to equilibrate to room temperature.
- 502 2. Add 24 mL of charcoal/dextran treated FBS, 10 mL L-glutamine, and 5 mL Pen-503 Strep to one 500 mL bottle of DMEM.
- 3. Label estrogen-free DMEM bottle as indicated in **Section 7.3**
- 505 Store at 2-8°C for no longer than six months or until the shortest expiration date of any media
- 506 component..

507 8.3 1X Trypsin Solution

- 1X trypsin solution is prepared by dilution from a 10X premixed stock solution. The 10X stock
- solution should be stored in 10 mL aliquots in a -20°C freezer.
- Procedure for making 100 mL of 1X trypsin:
- 1. Remove a 10 mL aliquot of 10X trypsin from -20°C freezer and allow to equilibrate to room temperature.
- 513 2. Aliquot 1 mL trypsin (10X) along with 9 mL of 1X PBS into ten 15 mL sterile centrifuge tubes.
- 515 3. Label 1X trypsin aliquots as indicated in **Section 7.3**
- 516 *IX Trypsin should be stored at -20°C*.

517 **8.4 1X Lysis Solution**

- Lysis solution is prepared by dilution from a 5X premixed stock solution. Both the 5X and 1X
- solutions can be repeatedly freeze-thawed.
- The procedure for making 10 mL of 1X lysis solution:
- 521 1. Thaw the 5X Promega Lysis solution and allow it to reach room temperature.
- 522 2. Remove 2 mL of 5X solution and place it in a 15 mL conical centrifuge tube.
- 3. Add 8 mL of distilled, de-ionized water to the conical tube.
- 524 4. Cap and shake gently until solutions are mixed.
- 525 Store at -20°C for no longer than 1 year from receipt.

526	8.5	Reconstituted Luciferase Reagent
527 528	Lucifera: substrate	se reagent consists of two components, luciferase buffer and lyophilized luciferase
529 530	_	term storage, unopened containers of the luciferase buffer and lyophilized luciferase can be stored at -70°C for up to one year.
531	To recon	stitute luciferase reagent:
532533		1. Remove luciferase buffer and luciferase substrate from -70°C freezer and allow them to equilibrate to room temperature.
534535		2. Add 10 mL of luciferase buffer solution to luciferase substrate container and swirl or vortex gently to mix; the Luciferase substrate should readily go into solution.
536		3. After solutions are mixed, aliquot to a 15mL centrifuge tube.
537		4. Store complete solution at −20°C.
538	Reconsti	tuted luciferase reagent is stable for up to 1 month at -20° C.
539	8.6	Reconstituted CellTiter-Glo® Reagent
540 541		r-Glo [®] reagent consists of two components, CellTiter-Glo [®] buffer and lyophilized c-Glo [®] substrate.
542543	_	term storage, unopened containers of the CellTiter-Glo [®] buffer and lyophilized c-Glo [®] substrate can be stored at -70°C for up to one year.
544	To recon	stitute CellTiter-Glo® reagent:
545 546		1. Remove CellTiter-Glo [®] buffer and CellTiter-Glo [®] substrate from -70°C freezer and allow them to equilibrate to room temperature.
547548549		2. Add CellTiter-Glo [®] buffer solution to CellTiter-Glo [®] substrate container and swirl or vortex gently to mix; the CellTiter-Glo [®] substrate should readily go into solution.
550		3. After solutions are mixed aliquot to a 15mL centrifuge tube.
551		4. Store complete solution at −20°C.
552	Reconsti	tuted CellTiter-Glo [®] reagent is stable for up to 3 months at – 20°C.
553 554	9.0	OVERVIEW OF PROPAGATION AND EXPERIMENTAL PLATING OF BG1Luc4E2 CELLS
555 556		Luc4E2 (BG-1) cells are stored in liquid nitrogen in 2 mL cryovials. BG-1 cells are a monolayer in tissue culture flasks in a dedicated tissue culture incubator at 37° C \pm

- 1°C, 90% \pm 5% humidity, and 5.0% \pm 1% CO₂/air. The cells should be examined, on a daily
- basis during working days, under an inverted phase contrast microscope and any changes in
- morphology and/or adhesive properties must be noted in the study notebook.
- Two T150 flasks containing cells at 80 to 90% confluence will usually yield a sufficient number
- of cells to fill four 96-well plates for use in experiments.

562 9.1 Procedures for Thawing Cells and Establishing Tissue Cultures

- Warm all of the tissue culture media and solutions to room temperature by placing them under
- the tissue culture hood several hours before use.
- All tissue culture media, media supplements, and tissue culture plasticware must be quality
- tested before use in experiments (Section 15.0).

567 9.1.1 Thawing Cells

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- 1. Remove a cryovial of frozen BG-1 cells from the liquid nitrogen flask.
- 569 2. Facilitate rapid thawing by loosening the top slightly (do not remove top) to release trapped gasses and retightening it. Roll vial between palms.
- 3. Use a micropipette to transfer cells to a 50 mL conical centrifuge tube.
- 4. Rinse cryovial twice with 1X PBS and add PBS rinse material to the conical tube.
- 5. Add 20 mL of RPMI to the conical tube.
 - 6. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
 - 7. Aspirate media from pellet and re-suspend it in 5 mL RPMI, drawing the pellet repeatedly through a 1.0 mL serological pipette to break up any clumps of cells.
 - 8. Transfer cells to a T25 flask, place them in an incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

580 9.1.2 Establishing Tissue Cultures

- Once cells have reached 80 to 90% confluence, transfer the cells to a T75 flask by performing,
- for example, the following steps:
- 1. Remove the T25 flask from the incubator.
- 584 2. Aspirate the RPMI, then add 5 mL 1X PBS, making sure that the cells are coated with PBS.
- 3. Aspirate 1X PBS, then add 1 to 2 mL 1X trypsin to the T25 flask, gently swirling the flask to coat all cells with the trypsin.
 - 4. Place the flask in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.

589 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the 590 hand. 591 6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, 592 593 then hit the flask again. 594 7. After cells have detached, add 5 mL PBS, and transfer the suspended cells to a 50 595 mL centrifuge tube. Wash the flask one additional time with 5 mL PBS. 596 Immediately add 20 mL RPMI to the conical tube to inhibit further cellular 597 digestion by residual trypsin. 598 9. Pellet the cells by centrifugation, as described in **Section 9.1.1**, and re-suspend the 599 cells in 10 mL RPMI medium. 600 10. Draw the pellet repeatedly through a 25 mL serological pipette to break up 601 clumps of cells. 602 11. Transfer cells to a T75 flask, then place the flask in an incubator (see conditions 603 in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs). 604 When cells have reached 80% to 90% confluence, transfer them into a T150 flask by performing. 605 for example, the following steps: 606 12. Remove the T75 flask from the incubator, aspirate the old media and add 5 mL 607 1X PBS. 13. Aspirate 1X PBS, add 2 mL of 1X trypsin to the flask, and place it in an incubator 608 609 (see conditions in **Section 9.0**) for 5 to 10 min. 610 14. Repeat steps 5 through 11 in Section 9.1.2, re-suspending the pellet in 20 mL of 611 RPMI. 612 15. Transfer cells to a T150 flask and place it in the incubator (see conditions in 613 **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs). 614 16. Remove the T150 flask from the incubator. 615 17. Aspirate the RPMI and add 5 mL 1X PBS. 616 18. Aspirate 1X PBS and add 3 mL 1X trypsin to the T150 flask, making sure that the 617 cells are coated with the trypsin. 618 19. Incubate cells in an incubator (see conditions in **Section 9.0**) for 5 to 10 min. 619 20. Detach cells by hitting the side of the flask sharply against the palm or heel of the

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hand.

621 21. Confirm cell detachment by examination under an inverted microscope. If cells 622 have not detached, return the flask to the incubator for an additional 2 minutes, 623 then hit the flask again. 624 22. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells 625 from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the 626 flask, swirl around the flask, and then transfer the PBS to the 50 mL conical tube. 627 23. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular 628 digestion by residual trypsin. 629 24. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge 630 for an additional 5 minutes. 631 25. Aspirate the media from the pellet and re-suspend it in 40 mL RPMI, drawing the 632 pellet repeatedly through a 25 mL serological pipette to break up any clumps of 633 cells. 634 26. Transfer 20 mL of cell suspension to each of two T150 flasks, place them in an 635 incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence 636 (approximately 48 to 72 hrs). 637 9.2 Ongoing Tissue Culture Maintenance, Conditioning in Estrogen-free Medium, and Plating Cells for Experimentation 638 639 The following procedure is used to condition the BG1Luc4E2 cells to an estrogen-free 640 environment prior to plating the cells in 96-well plates for analysis of estrogen dependent 641 induction of luciferase activity. 642 To start the tissue culture maintenance and estrogen-free conditioning, split the two T150 culture 643 flasks into four T150 flasks. Two of these flasks will be used for continuing tissue culture and 644 will use the RPMI media mentioned above. The other two flasks will be cultured in estrogen-free 645 DMEM for experimental use. Extra care must be taken to avoid contaminating the estrogen-free cells with RPMI 646 1. Remove both T150 flasks from the incubator. 647 648 2. Aspirate the medium and rinse the cells with 5 mL 1X PBS. 649 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask 650 to coat all cells with the trypsin. 651 4. Incubate cells in the incubator (see conditions in **Section 9.0**) for 5 to 10 min. 652 Detach cells by hitting the side of the flask sharply against the palm or heel of the 653 hand.

6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, then hit the flask again.
7. After cells have detached, add 5 mL 1X PBS to the first T150 flask and transfer the suspended cells to the second T150 flask.
8. Transfer the contents of both flasks to a 50 mL conical tube. Repeat step 7 with an

additional 5 mL 1X PBS and transfer to the 50 mL conical tube.

- 9. Immediately add 20 mL estrogen-free DMEM to the 50 mL conical tube to inhibit further cellular digestion by residual trypsin.
- 10. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
- 11. Aspirate media from pellet and re-suspend it in 4 mL estrogen-free DMEM, drawing the pellet repeatedly through a 1 mL serological pipette to break up clumps of cells.
- At this point, cells are ready to be divided into the ongoing tissue culture and estrogen-free conditioning groups.

670 9.2.1 <u>Ongoing Tissue Culture Maintenance</u>

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- 1. Add 20 mL RPMI to two T150 flasks.
 - 2. Add 220 µl G418 to the RPMI in the T150 flasks
- 3. Add 1 mL of cell suspension from **9.2** step **11** to each flask.
- 4. Place T150 flasks in tissue culture incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
 - 5. Tissue culture medium may need to be changed 24 hours after addition of G418 to remove cells that have died because they do not express reporter plasmid.
 - 6. G418 does not need to be added to the flasks a second time.
- 7. Repeat Section 9.2 steps 1-11 for ongoing tissue culture maintenance.

680 9.2.2 Conditioning in Estrogen-free Medium

- 1. Add 20 mL estrogen-free DMEM to two T150 flasks.
- 682 2. Add 150 μL G418 to the estrogen-free DMEM in the T150 flasks.
- 683 3. Add 1 mL of cell suspension from Section 9.2 step 11 to each flask.
- 4. Tissue culture medium may need to be changed 24 hours after addition of G418 to remove cells that have died because they do not express reporter plasmid.
 - 5. G418 does not need to be added to the flasks a second time.

687 688		6.	Place the T150 flasks in the incubator (see conditions in Section 9.0) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
689 690 691	9.2.3	<u>Plat</u> 1.	Remove the T150 flasks that have been conditioned in estrogen-free DMEM for 48 to 72 hours from the incubator.
692		2.	Aspirate the medium, then rinse the cells with 5 mL 1X PBS.
693 694		3.	Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask to coat all cells with the trypsin.
695		4.	Place the flasks in an incubator (see conditions in Section 9.0) for 5 to 10 min.
696 697		5.	Detach cells by hitting the side of the flask sharply against the palm or the heel of the hand.
698 699 700		6.	Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for 2 additional minutes, then hit the flask again.
701 702 703		7.	After cells have detached, add 5 mL 1X PBS and transfer the suspended cells from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the flask, gently swirl around the flask, and then transfer to the 50 mL conical tube.
704 705		8.	Immediately add 20 mL estrogen-free DMEM to each conical tube to inhibit further cellular digestion by residual trypsin.
706 707		9.	Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
708 709 710		10.	Aspirate the media from the pellet and re-suspend it in 20 mL DMEM, drawing the pellet repeatedly through a 25 mL serological pipette to break up any clumps of cells.
711 712 713		11.	Pipette 15 µL of the cell suspension into the "v" shaped slot on the hemocytometer. Ensure that the solution covers the entire surface area of the hemocytometer grid, and allow cells to settle before counting.
714		12	Using 100x magnification, view the counting grid.
715 716			The counting grid on the hemocytometer consists of nine sections, four of which are counted (upper left, upper right, lower left, and lower right, see Figure 9-1).
717			Each section counted consists of four by four grids. Starting at the top left and
718719			moving clockwise, count all cells in each of the four by four grids. Some cells will be touching the outside borders of the square, but only count those that touch

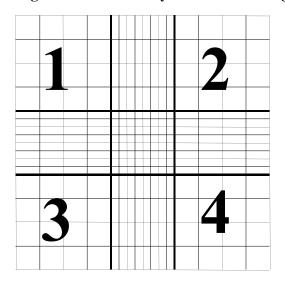
the top and right borders of the square. This value is then used in the calculation below to get to the desired concentration of 200,000 cells/mL.

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Figure 9-1 **Hemocytometer Counting Grid.**



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cells/mL)

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Concentration Final = 200,000 cells/mL

The volume of each square is 10⁻⁴ mL, therefore: Cells/mL=(average number per grid) x 10⁻⁴ mL x 1/(starting dilution).

Starting dilution: 20 mL (for T150 flasks)

- Harvested cells for a T150 flask are suspended in 20 mL of estrogen-free DMEM and sampled
- Example Calculation:
 - Grids 1, 2, 3, and 4 are counted and provide the following data:
 - o 50, 51, 49, and 50: average number of cells per grid is equal to 50.
- Cells/mL = 50 cells per grid $\div 10^{-4}$ mL volume of grid = 50 X 10^{4} cells/mL (or 500,000 734
- Total # of Cells Harvested = 500,000 cells/mL x 20 mL

for determination of concentration of cells/mL.

- Desired Concentration (or Concentration Final) = 200,000 cells/mL
- Formula: (Concentration Final x Volume Final = Concentration Initial x Volume Initial) 738

- 741 Concentration Initial = 500,000 cells/mL
- 742 Volume Initial = 20 mL
- 743 Volume Final to be solved for.

- Therefore: 200,000 cells/mL x Volume $_{\text{Final}} = 500,000 \text{ cells/mL x } 20 \text{ mL}$
- 746 Solving for Volume $_{Final}$ we find = 50 mL

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Therefore, add 30 mL of estrogen-free DMEM to the cell suspension for a total volume of 50 mL, which will yield the desired concentration of 200,000 cells/mL for plating.

750 751 14. This dilution scheme will give a concentration of 200,000 cells/mL. 200 μ L of this cell suspension is used for each well of a 96-well plate (i.e., 40,000 cells per well).

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15. Remove a 96-well plate from its sterile packaging. Use a repeater pipetter to pipette:

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200 μL of cell suspension into each well for range finder testing

756 757 200 μL of cell suspension into each well except the outside ring of wells for comprehensive testing and add 200 μL of estrogen-free DMEM to the outside ring of wells

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16. Incubate plate(s) in an incubator (see conditions in **Section 9.0**) for a minimum of 24 hours, but no longer than 48 hours before dosing.

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Two T150 flasks containing cells at 80% to 90% confluence will typically yield sufficient cells to fill four 96-well plates (not including the perimeter wells).

10.0 PREPARATION OF TEST SUBSTANCES

- The solvent used for dissolution of test substances is 100% DMSO. All test substances should be
- allowed to equilibrate to room temperature before being dissolved and diluted. Test substance
- solutions (except for reference standards and controls) should not be prepared in bulk for use in
- subsequent tests. Test substances are to be used within 24 hours of preparation. Solutions should
- not have noticeable precipitate or cloudiness.
- All information on weighing, solubility testing, and calculation of final concentrations for test
- substances, reference standards and controls is to be recorded in the study notebook.

771 **10.1 Determination of Test Substance Solubility**

- 772 1. Prepare a 100 mg/mL solution of the test substance in 100% DMSO in a 4 mL conical tube.
 - 2. Vortex to mix.
 - 3. If the test substance does not dissolve at 100 mg/mL, prepare a 10 mg/mL solution and vortex as above.
 - 4. If the test substance does not dissolve at 10 mg/mL solution, prepare a 1 mg/mL solution in a 4 mL conical tube and vortex as above.
 - 5. If the test substance does not dissolve at 1 mg/mL, prepare a 0.1 mg/mL solution in a 4 mL conical tube and vortex as above.
 - 6. Continue testing, using 1/10 less substance in each subsequent attempt until test substance is solubilized in DMSO.
- Once the test substance has fully dissolved in 100% DMSO, the solubility of the test substance must be determined in the 1% DMSO/99% estrogen-free DMEM mixture used for LUMI-
- 785 CELL® ER testing.

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 7. Add 4 μL of the highest concentration of the test substance/DMSO solution to a
 13 mm test tube.
 - 8. Add 400 µL estrogen-free DMEM to the test tube and vortex gently,
 - 9. If cloudiness or precipitate develop, vortex for up to 10 minutes.
- 790 10. If test substance has visible precipitate or is cloudy return to **10.1 step 7** to try the next lower concentration for the test substance.
- The Testing Facility shall forward the results from the solubility tests assay to the SMT through the designated contacts in electronic format and hard copy upon completion of testing.

794 10.2 Preparation of Reference Standards, Control and Test Substances

- 795 All "dosing solutions" of test substance concentrations are to be expressed as $\mu g/mL$ in the study
- notebook and in all laboratory reports.
- All information on preparation of test substances, reference standards and controls is to be
- recorded in the study notebook.
- 799 10.2.1 Preparation of Reference Standard and Positive Control Stock Solutions
- Stock solutions of E2 and methoxychlor are prepared in 100% DMSO and stored at room
- temperature for up to three years or until the expiration date listed in the certificate of analysis
- for that substance.

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- 803 10.2.1.1 *E2 Stock Solution*
- The final concentration of the E2 stock solution is $1.0 \times 10^{-2} \,\mu\text{g/mL}$. Prepare the E2 stock as
- 805 shown in **Table 10-1**.

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Table 10-1 Preparation of E2 Stock Solution

Step #	Action	DMSO	E2 Concentration
1	Make a 10 mg/mL stock solution in 100% DMSO in a 4mL vial.	-	10 mg/mL
2	Transfer 10 µL E2 solution from Step #1 to a new 4 mL vial.	Add 990 μL of 100% DMSO. Vortex to mix.	100 μg/mL
3	Transfer 10 µL E2 solution from Step #2 to a new 4mL vial.	Add 990 μL of 100% DMSO. Vortex to mix.	1 μg/mL
4	Transfer 10 µL E2 solution from Step #3 to a 13 mm test tube to create the working solution.	Add 990 μL of 100% DMSO. Vortex to mix.	0.1 μg/mL

- 807 10.2.1.2 Methoxychlor Stock Solution
- The final concentration of the methoxychlor stock solution is 313 μ g/mL.
- To prepare the methoxychlor stock solution, proceed as follows:
- 1. Make a 10 mg/mL stock solution of Methoxychlor in 100% DMSO in a 4 mL vial.
 - 2. Remove 94 µL of the methoxychlor solution and place it in a new 4 mL vial.
 - 3. Add 2.906 mL of 100% DMSO to the 4mL vial and gently vortex to mix.
- 814 10.2.2 <u>Preparation of Reference Standard and Positive Control Dosing Solutions for Range</u> 815 Finder Testing
- Range finder testing is conducted on 96-well plates using three concentrations of E2 in duplicate as the reference standard. Six replicate wells are used for the DMSO control. All wells on the 96 well plate are used during range finder testing.
- Store dosing solutions at room temperature. Use within 24 hours of preparation.
- 820 10.2.2.1 Preparation of E2 Reference Standard Dosing Solutions for Range Finder Testing
- In preparation for making E2 dosing solutions, label two sets of three glass 13 mm test tubes
- with the numbers one through three and place them in a test tube rack. Tube number 1 will
- contain the highest concentration of E2 (**Table 10-2**).

Table 10-2 Preparation of E2 Reference Standard Dosing Solution for Range Finder Testing

Tube Number	E2	Estrogen- free DMEM ¹	Final Volume	Final E2 Concentration
1	$4~\mu l$ of $1.0~x~10^{2}~\mu g/mL$ working solution	400 μL	404 μL	1.00 x 10 ⁻³ μg/mL
2	$8~\mu L$ of 1.0 x $10^{3}~\mu g/mL$ from Tube #1	400 μL	408 μL	2.00 x 10 ⁻⁵ μg/mL
3	$1~\mu L$ of $1.0~x~10^{-3}~\mu g/mL$ from Tube#1	4000 μL	4001 μL	1.00 x 10 ⁻⁶ μg/mL

Vortex all tubes to mix media and E2.

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10.2.2.2 Preparation of DMSO Control Dosing Solution for Range Finder Testing

1. Add 4 μL of 100% DMSO to six 13 mm tubes (solvent/negative controls).

2. Add 400 μL of estrogen-free DMEM to each tube and vortex vigorously.

10.2.3 <u>Preparation of Test Substance Dosing Solutions for Range Finder Testing</u>

Range finder experiments are used to determine the concentrations of test substance to be used during comprehensive testing. Agonist range finding for coded substances consists of six point, logarithmic serial dilutions run in duplicate.

Label two sets of six glass 13 mm test tubes with the numbers 1 through 6 and place them in a test tube rack. Tube number 1 will contain the highest concentration of test substance (**Table 10-3**).

Table 10-3 Preparation of Test Substance Dosing Solutions for Range Finder Testing

Tube Number	100% DMSO	Test Substance ¹	Transfer ²	Estrogen- free DMEM ³	Final Volume
1	-	4 μL of test substance solution from Section 10.1 step 14	4 μL	400 μL	404 μL
2	90 μL	10 μL of test substance solution from Section 10.1 step 14	4 μL	400 μL	404 μL
3	90 μL	10 μL from Tube #2	4 μL	400 μL	404 μL
4	90 μL	10 μL from Tube #3	4 μL	400 μL	404 μL
5	90 μL	10 μL from Tube #4	4 μL	400 μL	404 μL
6	90 μL	10 μL from Tube #5	4 μL	400 μL	404 μL

¹Vortex tubes #2 through 5 before removing test substance solution to place in the next tube in the series.

²Transfer test substance/DMSO solutions to a new set of 13 mm test tubes.

³Vortex all tubes to mix media and test substance solution.

Determination of whether a substance is positive in range finder testing and selection of starting concentrations for comprehensive testing will be discussed in **Section 12.0**.

10.2.4 <u>Preparation of Reference Standard and Positive Control Dosing Solutions for Comprehensive Testing</u>

Comprehensive testing is conducted on 96-well plates using 10 concentrations of E2 in duplicate as the reference standard. Four replicate wells for the DMSO control and three replicate wells for the methoxychlor control are included on each plate.

Store dosing solutions at room temperature. Use within 24 hours of preparation.

10.2.4.1 Preparation of E2 Reference Standard Dosing Solutions for Comprehensive Testing In preparation for making E2 double serial dilutions, label two sets of 11 glass 13 mm test tubes with the numbers 1 through 11 and place them in a test tube rack. Tube number 1 will contain the highest concentration of E2 (**Table 10-4**).

Table 10-4 Preparation of E2 Reference Standard Dosing Solution for Comprehensive Testing

Tube Number	100% DMSO	E2 ¹	Discard	Estrogen- free DMEM ²	Final Volume
1	-	4 μL of 1.0 x 10 ⁻² μg/mL working solution	-	400 μL	404 μL
2	4 μL	4 μL of 1.0 x 10 ⁻² μg/mL stock solution	-	400 μL	404 μL
3	4 μL	4 μL from Tube #2	-	400 μL	404 μL
4	4 μL	4 μL from Tube #3	-	400 μL	404 μL
5	4 μL	4 μL from Tube #4	-	400 μL	404 μL
6	4 μL	4 μL from Tube #5	-	400 μL	404 μL
7	4 μL	4 μL from Tube #6	-	400 μL	404 μL
8	4 μL	4 μL from Tube #7	-	400 μL	404 μL
9	4 μL	4 μL from Tube #8	Discard Tube #9	-	-
10	4 μL	4 μL from Tube #9	-	400 μL	404 μL
11	4 μL	4 μL from Tube #10	Remove and discard 4 µL from Tube #11	400 μL	404 μL

¹Vortex tubes #2 through 11 before removing E2 solution to place in the next tube in the series.

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²Vortex all tubes to mix media and E2.

- 861 10.2.4.2 Preparation of Methoxychlor Control Dosing Solution for Comprehensive Testing
- 1. Add 4 μL of the 313 μg/mL methoxychlor to three separate 13 mm tubes.
- 2. Add 400 μL of estrogen-free DMEM to each tube and vortex vigorously.
- 864 10.2.4.3 Preparation of DMSO Control Dosing Solution for Comprehensive Testing
 - 1. Add 4 μL of 100% DMSO to four 13 mm tubes (solvent/negative controls).
- 2. Add 400 μL of estrogen-free DMEM to each tube and vortex vigorously.

867 10.2.5 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

868 Comprehensive testing experiments are used to determine whether a substance possesses ER

agonist activity in the LUMI-CELL® ER test method. Agonist comprehensive testing for coded

substances consists of 11 point, double serial dilutions, with each concentration tested in

triplicate wells of the 96-well plate.

Start the 11-point serial dilution series at a single log dilution higher than the concentration

giving the highest adjusted RLU value during the range finder (e.g., if the highest adjusted RLU

value occurred at a concentration of 0.01 mg/mL, start the serial dilution at 0.1 mg/mL).

Label three sets of 11 glass 13 mm test tubes with numbers 1 through 11 and place them in a test

tube rack. Tube number 1 will contain the highest concentration of test substance (**Table 10-5**).

Table 10-5 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

Tube Number	100% DMSO	Test Substance ¹	Discard	Estrogen- free DMEM ²	Final Volume
1	-	4 μL of highest concentration of test substance solution	-	400 μL	404 μL
2	4 μL	4 μL of highest concentration of test substance solution	-	400 μL	404 μL
3	4 μL	4 μL from Tube #2	-	400 μL	404 μL
4	4 μL	4 μL from Tube #3	-	400 μL	404 μL
5	4 μL	4 μL from Tube #4	-	400 μL	404 μL
6	4 μL	4 μL from Tube #5	-	400 μL	404 μL
7	4 μL	4 μL from Tube #6	-	400 μL	404 μL
8	4 μL	4 μL from Tube #7	-	400 μL	404 μL
9	4 μL	4 μL from Tube #8	-	400 μL	404 μL
10	4 μL	4 μL from Tube #9	-	400 μL	404 μL
11	4 μL	4 μL from Tube #10	4 μL	400 μL	404 μL

¹Vortex tubes #2 through 11 before removing test substance solution to place in the next tube in the series.

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²Vortex all tubes to mix media and test substance solution.

GENERAL PROCEDURES FOR THE TESTING OF CODED SUBSTANCES 881 11.0 882 Range finder experiments are used to determine the concentrations of test substance to be used 883 during comprehensive testing. Comprehensive testing experiments are used to determine whether a substance possesses ER agonist activity in the LUMI-CELL® ER test method. 884 885 General procedures for range finder and comprehensive testing are nearly identical. For specific 886 details (such as plate layout) of range finder testing see Section 12.0. For specific details of 887 comprehensive testing, see Section 13.0. 888 11.1 **Application of Reference Standard, Controls, and Test Substances** 889 Remove the 96-well plates from the incubator, inspect them using an inverted 890 microscope. Only use plates in which the cells in all wells receive a score of 1 891 according to Table 11-1. 892 2. Remove medium by inverting the plate onto blotter paper. Gently tap plate against 893 the bench surface to remove residual liquid trapped in the wells. 894 3. Add 200 µL of medium, reference standard, control, or test substance to each well 895 (see Sections 12.0 and 13.0 for specific plate layouts). 896 Return plates to incubator and incubate (see Section 9.0 for details) for 19 to 24 897 hours to allow maximal induction of luciferase activity in the cells. <u>Preparation of Excel® Data Analysis Template</u> 898 11.1.1 In Excel®, open a new "AgICCVAMTemplate" and save it with the appropriate 899 900 project name as indicated in the NICEATM Style Guide. 901 2. Add appropriate information regarding the assay to the "Compound Tracking" 902 903 3. Enter substance testing information to the "List" page (i.e., Project /Sample ID, 904 Concentration, and Comments [or compound name]). This should populate the "Template", "Compound Mixing" and "Visual Inspection" tabs with the 905 906 appropriate information for the experiment. 907 4. Save the newly named project file. 908 Print out either the "List" or "Template" page for help with dosing the 96-well 909 plate. Sign and date the print out and store in study notebook. 910 11.2 **Visual Evaluation of Cell Viability** 911 19 to 24 hours after dosing the plate, remove the plate from the incubator and 912 remove the media from the wells by inverting the plate onto blotter paper. Gently

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tap plate against the bench surface to remove residual liquid trapped in the wells.

- 2. Use a repeat pipetter to add 50 μL 1X PBS to all wells. Immediately remove PBS by inversion.
 - 3. Using an inverted microscope, inspect all of the wells used in the 96-well plate and record the visual observations using the scores in **Table 11-1**.

Table 11-1 Visual Observation Scoring

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Viability Score	Brief Description ¹
1	Normal Cell Morphology and Cell Density
2	Altered Cell Morphology and/or Small Gaps between Cells
3	Altered Cell Morphology and/or Large Gaps between Cells
4	Few (or no) Visible Cells
1P	Score of 1 with Precipitate
2P	Score of 2 with Precipitate
3P	Score of 3 with Precipitate
4P	Score of 4 with Precipitate
5P	Unable to View Cells Due to Precipitate

¹Reference micrographs will be provided by NICEATM.

11.3 Lysis of Cells for LUMI-CELL® ER

- 1. Apply the reflective white backing tape to the bottom of the 96-well plate (this will increase the effectiveness of the luminometer).
- 2. Add 30 μ L 1X lysis reagent to the assay wells and place the 96-well plate on an orbital shaker for one minute.
- 3. Remove plate from shaker and measure luminescence (described in **Section 11.5**).

11.4 CellTiter-Glo® Assessment of Cell Viability

- When considered necessary, a quantitative evaluation of cell viability will be performed with the Promega CellTiter-Glo[®] assay system. CellTiter-Glo[®] uses luminescence as an indicator of the number of cells per plate and therefore must be conducted in parallel with the LUMI-CELL[®] ER test method (i.e., both test methods cannot be conducted on the same plate).
 - 1. Dose and incubate cells under the same conditions as for LUMI-CELL® ER.
 - 2. Remove plates from incubator and discard the medium by inverting the plate onto blotter paper. Gently tap plate against the bench surface to remove residual liquid trapped in the wells.
 - 3. Use a repeat pipettor to add 50 μL 1X PBS to all assay wells. Immediately remove PBS by inversion.

- 938 4. Examine all wells used under an inverted microscope. Make notes of any well 939 with codes described in Table 11-1. 940 5. Place white backing tape on the bottom of the 96-well plate. 941
 - 6. Add 100 μL estrogen-free DMEM to each well containing cells.
 - Add 100 µL CellTiter-Glo® reagent to each well containing cells. 7.
 - Place plate on an orbital shaker for 1 minute to induce cell lysis.
- 944 9. Incubate (see **Section 9.0** for details) for 10 min.
 - 10. Measure luminescence promptly as in **Section 11.5**. Do *not* add luciferase reagent to CellTiter-Glo® plates.

11.5 **Measurement of Luminescence**

- 948 Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and
- 949 with software that controls the injection volume and measurement interval. Light emission from
- 950 each well is expressed as RLU per well. The luminometer output is saved as raw data in an
- Excel® spread sheet. A hard copy of the luminometer raw data should be signed, dated and stored 951
- 952 in the study notebook.

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953 11.6 **Data Analysis**

- LUMI-CELL® ER uses an Excel® spreadsheet to collect and adjust the RLU values obtained 954
- from the luminometer and a GraphPad PRISM® template to analyze and graph data. The Excel® 955
- 956 spreadsheet subtracts background luminescence (average DMSO solvent control RLU value)
- 957 from test substance, reference standard and control RLU values. Plate induction is calculated
- 958 using these corrected RLU values. Test substance, reference standard, and control RLU values
- are then adjusted relative to the highest E2 reference standard RLU value, which is set to 10,000. 959
- After adjustment, values are transferred to GraphPad PRISM® for data analysis and graphing. 960

961 11.6.1 Correction and Adjustment of Luminometer Data

- The following steps describe the procedures required to populate the Excel® spreadsheet that has 962
- 963 been configured to collect and adjust the RLU values obtained from the luminometer.
 - 1. Open the raw data file and the corresponding experimental Excel® spreadsheet from **Section 11.1.1**.
 - 2. Copy the raw data using the Excel® copy function, then paste the copied data into cell C22 of the "RAW DATA" tab in the experimental Excel® spreadsheet using the Paste Special – Values command. This position corresponds to position B2 in the table labeled Table 1 in this tab.

3. Examine the DMSO data in Table 1 of the Excel® spreadsheet to determine 970 971 whether there are any potential outliers. See Section 11.6.2 for further explanation 972 of outlier determinations. 973 4. If an outlier is identified, perform the following steps to remove the outlier from 974 calculations: 975 correct the equation used to calculate DMSO background in Table 1 976 (e.g., if outlier is located in cell G24, adjust the calculation in cell H42 to 977 read = AVERAGE(F24.H24,I24)978 then correct the equation used to calculate the average DMSO value in 979 Table 2 (e.g., following the above example, adjust cell M44 to read 980 =AVERAGE(F36,H36,I36)) 981 then correct the equation used to calculate the standard deviation of the 982 DMSO value in Table 2 (e.g., following the above example, adjust cell M45 to read =STDEV(F36,H36,I36)) 983 5. Excel® will automatically subtract the background (the average DMSO control 984 985 value) from all of the RLU values in Table 1 and populate Table 2 with these adjusted values. 986 987 6. To calculate plate induction, identify the cell containing the E2 replicate that has 988 the highest RLU value and the cell containing the RLU values for the same concentration in the corresponding E2 replicate (e.g., if the highest RLU value for 989 990 E2 is located in cell E23, the corresponding cell would be E22). 991 7. Click into cell D16 and enter the cell number from the previous step into the 992 numerator. 993 8. Click on the "ER Agonist Report" tab. 994 The data for the E2 reference standard, methoxychlor, and DMSO replicates 995 populate the left portion (columns A - F) of the spreadsheet. The data is automatically placed in an Excel® graph. 996 997 10. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell 998 D2 of "ER Agonist Report" tab and check the formula contained within that cell. 999 The divisor should be the cell number of the cell containing the highest averaged 1000 E2 RLU value (column E). 1001 11. Use the forecast feature in Excel[®] to calculate an EC₅₀. To use forecast: 1002 Calculate the midpoint by subtracting the lowest RLU from the highest 1003 and dividing by two.

- Forecast the EC50 value using the formula

 (FORECAST(x,known_y's,known_x's), where x = the data point for
 which you wish to predict a value [i.e., the median RLU], known y's =
 the three RLU values that constitute the middle of the linear portion of
 the concentration-response curve, and known x's = the concentrations
 corresponding to the known y's).
 - 12. Copy the data into GraphPad PRISM[®] for graphing as indicated in the NICEATM PRISM[®] user's guide.

1012 11.6.2 Determination of Outliers

The Study Director will use good statistical judgment for determining "unusable" wells that will be excluded from the data analysis and will provide an explanation in the study notebook for any excluded data. This judgment for data acceptance will include Q test analysis.

1016 The formula for the Q test is:

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 $\frac{Outlier - Nearest\ Neighbor}{Range\ (Highest - Lowest)}$

where the outlier is the value proposed for exclusion, the nearest neighbor is the value closest to the outlier, and the range is the range of the three values (Q values for samples sizes from 3 to 10 are provided in Table 11-2). For example, if the value of this ratio is greater than 0.94 (the Q value for the 90% confidence interval for a sample size of three) or 0.76 (the Q value for the 90% confidence interval for a sample size of four), the outlier may be excluded from data analysis.

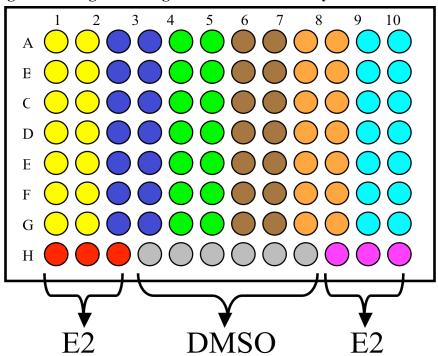
Table 11-2 Q Test Values

Number Of Observations	Q Value
2	-
3	0.94
4	0.76
5	0.64
6	0.56
7	0.51
8	0.47
9	0.44
10	0.41

For E2 reference standard replicates (sample size of two), any adjusted RLU value for a replicate at a given concentration of E2 is considered and outlier if its value is more than 20% above or below the adjusted RLU value for that concentration in the historical database.

1028	11.6.3	Acceptance Criteria
1029	Accepta	nce or rejection of a test is based on evaluation of reference standard and control results
1030	from eac	ch experiment conducted on a 96-well plate. Results are compared to quality controls
1031	(QC) for	r these parameters derived from the historical database, which are summarized below.
1032		• Induction: Plate induction, as measured by dividing the averaged highest E2
1033		reference standard RLU value by the averaged DMSO control RLU value, must
1034		be greater than three-fold.
1035		• Reference standard results: Calculated E2 reference standard EC ₅₀ values must be
1036		within 2.5 times the standard deviation of the historical database EC ₅₀ mean
1037		value.
1038		• Solvent control results: Solvent control RLU values must be within 2.5 times the
1039		standard deviation of the historical solvent control mean RLU value.
1040		• Positive control results: Methoxychlor control RLU values must be within 2.5
1041		times the standard deviation of the historical methoxychlor control mean RLU
1042		value.
1043	An expe	eriment that fails any single acceptance criterion will be discarded and repeated.
1044	12.0	RANGE FINDER TESTING
1045	Agonist	range finding for coded substances consists of six-point, logarithmic serial dilutions,
1046	with eac	ch concentration tested in duplicate wells of the 96-well plate. Figure 12-1 contains a
1047	template	e for the plate layout to be used in agonist range finder testing.
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Figure 12-1 Agonist Range Finder Test Plate Layout



- Three Point E2 Reference Standard Replicate 1
- Three Point E2 Reference Standard Replicate 2
- DMSO Control (1% v/v)
- Range Finder for Sample #2
- Range Finder for Sample #1
- Range Finder for Sample #3
- Range Finder for Sample #4
- Range Finder for Sample #5
- Range Finder for Sample #6
- Evaluate whether range finder experiments have met the acceptance criteria (see Section 11.6.3)
- and graph the data as described in the NICEATM PRISM® users guide.
- To determine starting concentrations for comprehensive testing use the following criteria:

- If there are no points on the test substance concentration curve that are above the line representing the mean plus three times the standard deviation of the DMSO control, the highest concentration used in comprehensive testing is the limit dose or the maximum soluble dose.
- If there are points on the test substance concentration curve that are above the line representing the mean plus three times the standard deviation of the DMSO control, select a concentration that is a single log dilution higher than the concentration giving the highest adjusted RLU value in the range finder, and use that as the highest concentration for comprehensive testing.
- If a substance exhibits a biphasic concentration curve, the range finder experiment should be repeated unless the proposed concentration range for the comprehensive studies will include all concentrations of the biphasic region in the range finding study. If the range finder experiment is repeated and the substance still exhibits a biphasic concentration curve, comprehensive testing must be conducted on the peak of the biphasic curve at the lowest test substance concentration. If the substance is negative at this lowest concentration, then test at the higher concentration. For either peak of the concentration curve, select a concentration that is a single log dilution higher than the concentration giving the highest adjusted RLU value in the range finder and use that as the highest concentration for comprehensive testing.

13.0 COMPREHENSIVE TESTING

Agonist comprehensive testing for coded substances consists of 11-point, double serial dilutions, with each concentration tested in triplicate wells of the 96-well plate. **Figure 13-1** contains a template for the plate layout to be used in agonist comprehensive testing.

Figure 13-1 Agonist Comprehensive Test Plate Layout

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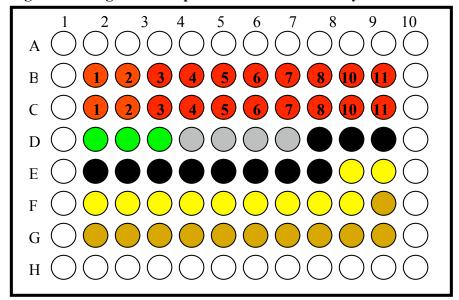
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- E2 Reference Standard Dose Response Curve Note: #9 in dilution series has been removed.
- Methoxychlor Control (3.13 μg/mL)
- O DMSO Control (1% v/v)
- Comprehensive Dose Response Sample #1, Replicate #1
- Comprehensive Dose Response Sample #1, Replicate #2
- Comprehensive Dose Response Sample #1, Replicate #3
- - Media only wells, not used for assay

Evaluate whether comprehensive experiments have met acceptance criteria (see **Section 11.6.4**) and graph the data as described in the NICEATM PRISM[®] users guide.

- If the substance has been tested up to the limit dose or the maximum soluble dose, without causing a significant decrease in cell viability, and there are no points on the concentration curve that are above the line indicating the mean plus three times the standard deviation of the DMSO control, the substance is considered negative for agonism.
- If the substance has a positive response (See **Section 6.0**) at any concentration, the substance is considered positive for agonism.

1087 14.0 USE OF THE HISTORICAL DATABASE TO GENERATE QC CHARTS

- The historical database is maintained in order to ensure that the test method is functioning
- properly. The historical database is maintained as an Excel® spreadsheet that is separate from the
- spreadsheets used to report the data for individual experiments. The controls used to develop the
- historical database are used as one of the criteria for determining a valid test.
- Results collected during Phase I will be compared to historical control data established during
- the LUMI-CELL® ER Protocol Standardization Study. Reference standard and control data
- 1094 collected during Phase I will be used to compile the initial historical database. Reference
- standard and control data collected during Phase IIa will be added to the historical database
- 1096 compiled in Phase I, and this combined historical database will be used to establish acceptance
- 1097 criteria for Phase II. Reference standard and control data collected during Phase IIb will be added
- to the historical database compiled in Phases I and IIa and this combined historical database will
- be used to establish acceptance criteria for Phases III and IV.

14.1 LUMI-CELL® ER Agonist QC Charts

- 1. Open the Excel® spreadsheet labeled LUMIAgonistQC.
- 2. Save this sheet under a new name, adding the laboratory designator to the file name (e.g., for Laboratory A, the new name would be ALUMIAgonistQC).

1104 14.1.1 Methoxychlor Control

- 1. Open the Excel[®] spreadsheet from **Section 14.1 step 2**.
 - 2. Click on the methoxychlor tab and enter the date, plate number (name), and average 2.5 x 10⁻⁵ μg RLU value for E2 (data located in column F on the "List" tab of the agonist report file).
 - 3. Enter the three values for methoxychlor into column D.
 - 4. The mean and 2.5 times the standard deviation plus (and minus) the mean are calculated automatically.
 - 5. Check the scatter charts tab to see if the average value for methoxychlor falls within the 2.5 times the standard deviation (**Figure 14-1**). If the average value falls within the 2.5 times the standard deviation area, methoxychlor passes QC. If the average value falls outside of the 2.5 times the standard deviation area, methoxychlor fails QC and the experiment must be repeated.
- Acceptance or rejection of the methoxychlor control data is based on whether the data for a given experiment falls within 2.5 times the standard deviation from the historical mean RLU value.

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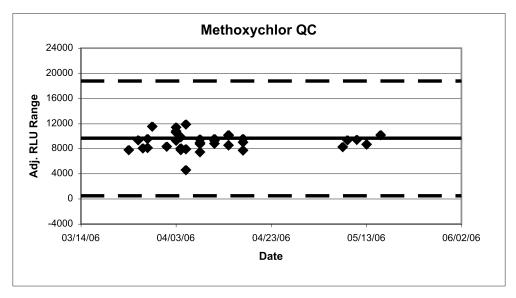
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Figure 14-1 Example Scatter Chart of the Methoxychlor Control QC^{1,2,3}



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1121 ¹Each point represents a single experiment.

²The solid line represents the historical mean RLU value for the methoxychlor control.

³The two dashed lines represent the historical mean RLU value for the methoxychlor control plus and minus 2.5 times the standard deviation from the historical mean.

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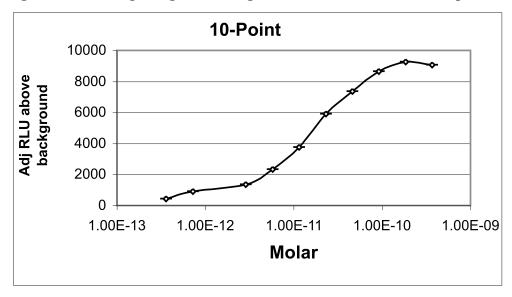
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14.1.2 <u>10-Point E2 Reference Standard QC</u>

- 1. Enter the experiment date and name, and copy and paste the adjusted RLU values for E2 into the appropriate slots in the tab labeled E2 Standard Curve.
- 2. The E2 standard curve is automatically graphed to ensure a normal sigmoidal shape (see **Figure 14-2** for an example curve).

Figure 14-2 Example Figure of a Sigmoidal E2 Concentration Response Curve¹



¹The line represents the averaged E2 values for a single experiment.

14.1.3 EC₅₀ Tracking Data

- 1. Enter the date and plate ID into the first two columns of the EC₅₀ Tracking Data tab.
- 2. Link the EC_{50} data from the 10-point E2 Curve QC tab to the column to the right of the plate information.
- 3. Column E calculates the percent deviation from the historical database EC_{50} value.
- 4. The mean and 2.5 times the standard deviation plus (and minus) the mean for the EC_{50} deviation are calculated automatically.
- 5. Check the Scatter Charts tab to see whether the experimental EC₅₀ value falls within the 2.5 times the standard deviation (**Figure 14-3**). If the value falls within the 2.5 times the standard deviation area, it passes QC. If the value does not fall within the 2.5 times the standard deviation, it fails QC and the experiment must be repeated.

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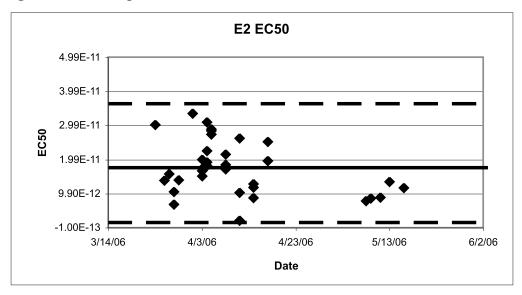
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Figure 14-3 Example Scatter Chart of the E2 EC₅₀ Control QC^{1,2,,3}



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1150 ¹Each point represents a single experiment.

1151 The solid line represents the historical mean RLU value for the E2 EC50.

³The two dashed lines represent the historical mean RLU value for the E2 EC50

control plus and minus 2.5 times the standard deviation from the historical mean.

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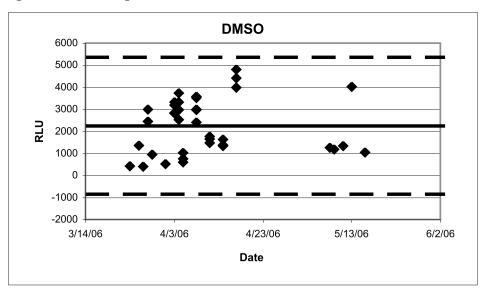
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1168 1169 Acceptance or rejection of E2 EC_{50} data is based on whether the data falls within 2.5 times the standard deviation from the mean for the historical E2 EC_{50} RLU value.

14.1.4 DMSO Control

- 1. The date and experiment name should populate automatically
- 2. Enter all of the DMSO values from Table 1 on the "Raw Data" tab on the Excel® spreadsheet which passed the outlier test, into the areas marked DMSO 1, DMSO 2, DMSO 3, and DMSO 4.
- 3. The average RLU value for DMSO is then calculated under the "mean" column.
- 4. The mean and 2.5 times the standard deviation plus (and minus) the mean for the DMSO deviation are calculated automatically.
- 5. Check the Scatter Charts tab to see whether the average value for DMSO falls within 2.5 times the standard deviation (**Figure 14-4**) from the mean. If the value falls within the 2.5 times the standard deviation area, the DMSO passes QC. If the value does not fall within the 2.5 times the standard deviation, it fails QC and the experiment must be repeated..

Figure 14-4 Example Scatter Chart of the DMSO Control QC^{1,2,3}



¹Each point represents a single experiment.

²The solid line represents the historical mean RLU value for the DMSO control.

1174 The two dashed lines represent the historical mean RLU value for the DMSO

control plus and minus 2.5 times the standard deviation from the historical mean.

Acceptance or rejection of the DMSO control data is based on whether the data for a given experiment falls within 2.5 times the standard deviation from the historical mean RLU value.

1179 14.1.5 Induction

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1180 Enter the induction value from the "Raw Data" tab on the Excel® spreadsheet. If the value is

greater than or equal to 3, the experiment passed QC. An induction value of less than 3 fails

induction QC and the experiment must be repeated.

15.0 OUALITY TESTING OF MATERIALS

All information pertaining to the preparation and testing of media, media supplements, and other materials should be recorded in the Study Notebook.

15.1 Tissue Culture Media

- Each lot of tissue culture medium must be tested in a single growth flask of cells before use in
- ongoing tissue culture or experimentation (**note**: each bottle within a given lot of
- 1189 Charcoal/Dextran treated FBS must be tested separately).

- Draft NICEATM LUMI-CELL® Protocol Standardization Report: Appendix B 06 April 2007 DO NOT CITE, QUOTE, OR DISTRIBUTE 1. Every new lot of media (RPMI and DMEM) and media components (FBS. 1190 1191 Charcoal/Dextran treated FBS, and L-glutamine) must first be tested on the LUMI-CELL® ER assay prior to being used in any GLP acceptable assays. 1192 1193 2. Add 4 µL of DMSO (previously tested) into four separate 13 mm tubes. 1194 3. Add 400 µL media (to be tested) to the same tubes. 1195 Dose an experimental plate as in **Section 12.0**, treating the media being tested as a 1196 test substance. 1197 5. Analyze 96-well plate as described in **Section 12.0**, comparing the data from the 1198 DMSO controls made using previously tested tissue culture media to the new 1199 media being tested. 1200 6. Use the QC charts to determine if the new media with DMSO lies within 2.5 1201 standard deviation of the mean for the media. If the RLU values for the new 1202 media with DMSO lie within 2.5 standard deviation of the mean for the historical 1203 data on DMSO, the new lot of media is acceptable. If the RLU values for the new media with DMSO do not lie within 2.5 standard deviation of the mean for the 1204 1205 historical data the new lot may not be used in the assay. 1206
 - 7. Note date and lot number in study notebook.
 - 8. If the new bottle passes the QC as described in **Section 15.1 step 6**, apply the media to a single flask cells and observe the cells growth and morphology over the following 2-3 days. If there is no change in growth or morphology, the new media is acceptable for use.

15.2 G418

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- 1. New lots of G418 must first be tested on the LUMI-CELL® ER assay prior to being used in any GLP acceptable assays.
- 2. Add 220 µL of G418 (previously tested) to a single flask containing cells growing in RPMI.
- 3. Add 220 µL of G418 (to be tested) to a different flask containing cells growing in RPMI.
- 4. Observe cellular growth and morphology in both tissue culture flasks over a 48 to 72 hour period. If there are no differences in observed growth rate and morphology between the two flasks, the new G418 lot is acceptable.
- 5. If cellular growth is decreased, or the cells exhibit abnormal morphology, the new lot of G418 is not acceptable.
- 6. Note date and lot number in study book.

1224 15.3 **DMSO** Every new bottle of DMSO must be tested on the LUMI-CELL® ER assay prior 1225 1226 to use in any GLP acceptable assays. 1227 2. Add 4 µL of DMSO (to be tested) into four separate 13 mm tubes. 1228 3. Add 400 µL media (previously tested) the same tubes. 1229 4. Dose an experimental plate as in **Section 12.0**, treating the media being tested as a 1230 test substance. 1231 5. Analyze 96-well plate as described in **Section 12.0**, comparing the data from the 1232 DMSO controls made using previously tested tissue culture media to the new 1233 media being tested. 1234 6. Use the QC charts to determine if the new media with DMSO lies within 2.5 1235 standard deviation of the mean for the media. If the RLU values for the new 1236 media with DMSO lie within 2.5 standard deviation of the mean for the historical 1237 data on DMSO, the new lot of media is acceptable. If the RLU values for the new 1238 media with DMSO do not lie within 2.5 standard deviation of the mean for the 1239 historical data the new lot may not be used in the assay. 7. Use the QC charts to determine if the new DMSO lies within 2.5 standard 1240 1241 deviation of the mean for DMSO background. If the RLU for the new DMSO 1242 does lie within 2.5 standard deviation of the mean for the historical data on DMSO, then the new bottle of DMSO is acceptable; otherwise the new bottle may 1243 not be used in the assay. 1244 1245 8. Note the date, lot number, and bottle number in study book. 1246 9. If no DMSO has been previously tested, test several bottles as described in 1247 Section 15.3, and determine whether any of the bottles of DMSO have a lower 1248 average RLU than the other bottle(s) tested. Use the DMSO with the lowest 1249 average RLU for official experiments.

15.4 Plastic Tissue Culture Materials

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- 1. Grow one set of cells, plate them for experiments on plastic ware from the new lot and one set of cells in the plastic ware from a previous lot, and dose them with E2 reference standard and controls.
- 2. Perform the LUMI-CELL® ER experiment with both sets of cells.
- 3. If all of the analysis falls within acceptable QC criteria, then the new manufacturer's products may be used.

1257	16.0 REFERENCES
1258	ICCVAM. 2001. Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses
1259	for Acute Toxicity. NIH Pub. No. 01-4500. Research Triangle Park, NC: National Institute of
1260	Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/invidocs/
1261	guidance/iv_guide.pdf [accessed 31 August 2006]

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7	Appendix C
8	Protocol for the LUMI-CELL® Estrogen Receptor (ER) Transcriptional Activation Test
9	Method for the Detection of ER Antagonists

 $\label{eq:condition} \textit{Draft NICEATM LUMI-CELL}^{\circledR} \textit{Protocol Standardization Report: Appendix C DO NOT CITE, QUOTE, OR DISTRIBUTE}$

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Developed by:

Xenobiotic Detection Systems, Inc.

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Durham, NC 27704

	Draft NICEATM LUMI-CELL $^{\$}$ Protocol Standardization Report: Appendix C DO NOT CITE, QUOTE, OR DISTRIBUTE	06 April 2007
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117	117 LIST OF ACRONYMS AND ABBREVIATIONS		
118	13 mm test tube	13 x 100 mm glass test tubes	
119	Absolute IC ₅₀ Value	Concentration of a substance that decreases the measured	
120		activity in an antagonist assay to 50% of the maximum	
121		activity induced by the reference substance	
122	CASRN	Chemical Abstracts Service Reference Number	
123	DMEM	Dulbecco's Modification of Eagle's Medium	
124	DMSO	Dimethyl Sulfoxide	
125	DMSO Control	1% v/v dilution of DMSO in tissue culture media	
126		used as a vehicle control	
127	E2	17β-estradiol	
128	E2 Control	$2.5 \times 10^{-5} \mu g/mL$ E2 used as a control.	
129	ER	Estrogen Receptor	
130	Estrogen-free DMEM	DMEM (phenol red free), supplemented with 1 %	
131		Penicillin/Streptomycin, 2 % L-Glutamine, and 5%	
132		Charcoal-dextran treated FBS	
133	FBS	Fetal Bovine Serum	
134	Flavone/E2 Control	$25 \mu g/mL flavone + 2.5 \times 10^{-5} \mu g/mL E2$,	
135		used as a positive control.	
136	G418	Gentamycin	
137	GLP	Good Laboratory Practice	
138	PBS	Phosphate Buffered Saline	
139	Ral/E2 Reference Standard	Nine point dilution of raloxifene HCl + $2.5 \times 10^{-5} 17\beta$ -	
140		estradiol reference standard for the LUMI-CELL® ER	
141		antagonist assay	
142	Relative IC ₅₀ Value	Concentration that produces a half-maximal response as	
143		calculated using the four parameter Hill function.	
144	RLU	Relative Light Units	
145	RPMI	RPMI 1640 growth medium	
146	SMT	Study Management Team	
147	SOP	Standard Operating Procedure	
148	TA	Transcriptional Activation	
149	T25	25 cm ² tissue culture flask	
150	T75	75 cm ² tissue culture flask	
151	T150	150 cm ² tissue culture flask	
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1 1.0 PURPOSE

- 2 This protocol is designed to evaluate coded test substances for potential estrogen receptor (ER)
- antagonist activity using the LUMI-CELL® ER assay.

4 2.0 SPONSOR

- 5 The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative
- 6 Toxicological Methods (NICEATM), P.O. Box 12233 Research Triangle Park, NC 27709
- 8 William S. Stokes, DVM, DACLAM
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59 **2.1 Substance Inventory and Distribution Management**

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67	3.0	DEFINITIONS	
68		• Dosing Solution: The test substance, control substance, or reference standard	
69		solution which is to be placed into the tissue culture wells for experimentation.	
70		• Raw Data: Raw data includes information that has been collected but not	
71		formatted or analyzed, and consists of the following:	
72		 Data recorded in the Study Notebook 	
73		 Computer printout of initial luminometer data 	
74		 Other data collected as part of GLP compliance, e.g.: 	
75		 Equipment logs and calibration records 	
76		 Test substance and tissue culture media preparation logs 	
77		 Cryogenic freezer inventory logs 	
78	• Soluble: Test substance exists in a clear solution without visible cloudiness or		
79	precipitate.		
80		• Study Notebook: The study notebook contains recordings of all activities related	
81		to the conduct of the LUMI-CELL® ER TA antagonist assay.	
82		• Test Substances: Substances supplied to the testing laboratories that are coded	
83		and distributed such that only the Project Officer, Study Management Team	
8485		(SMT), and the Substance Inventory and Distribution Management have	
86		knowledge of their true identity. The test substances will be purchased, aliquoted coded, and distributed by the Supplier under the guidance of the NIEHS/NTP	
87		Project Officer and the SMT.	
88	4.0	TESTING FACILITY AND KEY PERSONNEL	
89	4.1	Testing Facility	
90	Xenobiotic Detection Systems, Inc. (XDS), 1601 E. Geer St., Durham, NC 27704		
91	4.2	Key Personnel	
92		• Study Director: John Gordon, Ph.D.	
93		Laboratory Technician(s): Cynthia Matherly	
94		• Scientific Advisor: Mike Denison, Ph.D.	
95		Quality Assurance Director: Andrew Chu	
96		Safety Manager: George Clark	
97		• Facility Management: George Clark, Ph.D., Robert Clark, Tina Ginter	

• Laboratory Director: Andrew Chu

99 5.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

100 5.1 Test Substances

- Test substances are coded and will be provided to participating laboratories by the Substance
- 102 Inventory and Distribution Management team.

103 **5.2 Controls**

- 104 Controls for the ER antagonist protocol are as follows:
- 105 Vehicle control (dimethyl sulfoxide [DMSO]): 1% v/v dilution of DMSO (CASRN 67-68-5)
- diluted in tissue culture media.
- 107 Ral/E2 reference standard for range finder testing: Three concentrations (1.25 x 10⁻¹, 1.25 x 10³,
- and 5.00 x 10⁻⁵ µg/mL) of raloxifene HCl (raloxifene, CASRN 84449-90-1) plus a fixed
- concentration (2.5 x 10^{-5} µg/mL) of 17 β -estradiol (E2, CASRN: 50-28-2).
- 110 Ral/E2 reference standard for comprehensive testing: A serial dilution of raloxifene HCl
- (raloxifene, CASRN 84449-90-1) plus a fixed concentration (2.5 x 10^{-5} µg/mL) of 17β -estradiol
- 112 (E2, CASRN: 50-28-2), consisting of nine concentrations of Ral/E2 in duplicate wells.
- 113 E2 control: 17β-estradiol, CASRN: 50-28-2, 2.5 x 10⁻⁵ μg/mL in tissue culture media used as a
- base line negative control.
- 115 Flavone/E2 Control: Flavone, CASRN 525-82-6, 25 μg/mL, with E2 2.5 x 10⁻⁵ μg/mL in tissue
- culture media used as a weak positive control.

117 6.0 OVERVIEW OF GENERAL PROCEDURES FOR ANTAGONIST TESTING

- All experimental procedures are to be carried out under aseptic conditions and all solutions,
- glassware, plastic ware, pipettes, etc., shall be sterile. All methods and procedures shall be
- documented in the study notebook.
- 121 Antagonist range finder testing is conducted on 96-well plates using three concentrations of
- Ral/E2 (1.25 x 10^{-1} , 1.25 x 10^{-3} , and 5.00 x 10^{-5} µg/mL raloxifene with 2.50 x 10^{-5} µg/mL E2) in
- duplicate as the reference standard, with three replicate wells for the E2 and DMSO controls.
- Range finder testing uses all wells of the 96-well plate.
- 125 Comprehensive testing is conducted on 96-well plates using nine concentrations of Ral/E2 in
- duplicate as the reference standard (**Table 6-1**). Three replicate wells for the DMSO control,

Flavone/E2 and E2 controls are included on each plate. In order to avoid edging effects³, wells on the perimeter of the plate are not used for experiments. These wells should contain tissue culture media only.

Table 6-1 Concentrations of Ral/E2 Reference Standard Used in Comprehensive Testing

Raloxifene Concentrations ¹	E2 Concentrations
1.25 x 10 ⁻²	2.5 x 10 ⁻⁵
6.25 x 10 ⁻³	2.5 x 10 ⁻⁵
3.13 x 10 ⁻³	2.5 x 10 ⁻⁵
1.56 x 10 ⁻³	2.5 x 10 ⁻⁵
7.81 x 10 ⁻⁴	2.5 x 10 ⁻⁵
3.91 x 10 ⁻⁴	2.5 x 10 ⁻⁵
1.95 x 10 ⁻⁴	2.5 x 10 ⁻⁵
9.77 x 10 ⁻⁵	2.5 x 10 ⁻⁵
4.88 x 10 ⁻⁵	2.5 x 10 ⁻⁵

¹Concentrations are presented in µg/mL.

Visual observations for cell viability are conducted for all experimental plates just prior to LUMI-CELL® ER evaluation. CellTiter-Glo® based cell viability testing (when used) is conducted concurrently in parallel plates on the same day, as outlined in **Section 11.4**.

Luminescence data, measured in relative light units (RLUs), is corrected for background luminescence by subtracting the mean RLU value of the vehicle control (DMSO) wells from the RLU measurements for each of the other wells of the 96-well plate. Data is then transferred into Excel® data management spreadsheets and GraphPad PRISM® 4.0 statistical software, graphed, and evaluated for a positive or negative response as follows:

- A response is considered positive for antagonist activity when the average adjusted RLU for a given concentration is less than the mean RLU value minus three times the standard deviation for the E2 control.
- Any luminescence at or above this threshold is considered a negative response.

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³Edging effects are variations in response seen in the outermost wells in a cell culture plate. These variations are believed to be due to variations in temperature, evaporation, etc., that may occur in these wells that would ultimately affect cellular growth and health (ICCVAM, 2001).

- For substances that are positive at one or more concentrations, the concentration of test substance
- that causes a half-maximal response (the relative IC_{50}) is calculated using a Hill function
- analysis. The Hill function is a four-parameter logistic mathematical model relating the
- substance concentration to the response (typically following a sigmoidal curve) using the
- equation below

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(logIC50-X)HillSlope}}$$

- where Y = response (i.e., relative light units); X = the logarithm of concentration; Bottom = the
- minimum response; Top = the maximum response; $log IC_{50}$ = the logarithm of X as the response
- midway between Top and Bottom; and HillSlope describes the steepness of the curve. The model
- 155 calculates the best fit for the Top, Bottom, HillSlope, and IC₅₀ parameters. See **Section 13.6.5** for
- more details.
- 157 Acceptance or rejection of a test is based on evaluation of reference standard and control results
- 158 from each experiment conducted on a 96-well plate. Results for these controls are compared to
- historical results compiled in the historical database, as seen in **Section 16.0**.

160 **6.1** Range Finder Testing

- Antagonist range finding for coded substances consists of a six point, logarithmic serial dilution
- using duplicate wells per concentration. Concentrations for comprehensive testing are selected
- based on the response observed in range finder testing. If necessary, a second range finder test
- can be conducted to clarify the optimal concentration range to test (see **Section 14.0**).

165 **6.2** Comprehensive Testing

- 166 Comprehensive antagonist testing for coded substances consists of 11 point, double serial
- dilutions, with each concentration tested in triplicate wells of the 96-well plate. Three separate
- experiments are conducted for comprehensive testing on three separate days, except during
- Phases III and IV of the validation effort, in which comprehensive testing experiments are
- 170 conducted once (see **Section 15.0**).

171 7.0 MATERIALS FOR LUMI-CELL® ER ANTAGONIST TESTING

- 172 This section provides the materials needed to conduct LUMI-CELL® ER testing, with associated
- brand names/vendors⁴ in brackets.

⁴Brand names and vendors should not be considered an endorsement by the U.S. Government or any member of the U.S. Government; such information is provided as examples.

174 7.1 **BG1Luc4E2** Cells: 175 Human ovarian cancer cell line stably transfected with a plasmid containing an estrogen response 176 element (Figure 7-1) [XDS]. 177 Figure 7-1 pGudLuc7.ERE Plasmid. 178 179 180 7.2 **Technical Equipment:** 181 All technical equipment may be obtained from Fisher Scientific International, Inc. (Liberty Lane 182 Hampton, NH, USA 03842). Equivalent technical equipment from another commercial source 183 can be used. 184 Analytical balance (Cat. No. 01-910-320) 185 Berthold Orion 1 Microplate Luminometer [Berthold CatNo.: Orion 1 MPL3] or 186 equivalent and dedicated computer 187 Biological safety hood, class II, and stand (Cat. No. 16-108-99) 188 Centrifuge (low speed, tabletop with swinging bucket rotor) (Cat. No. 04-978-50 189 centrifuge, and 05-103B rotor) 190 Combustion test kit (CO₂ monitoring) (Cat. No. 10-884-1) 191 Drummond diaphragm pipetter (Cat. No. 13-681-15) 192 Freezers, -20°C (Cat. No. 13-986-150), and -70°C (Cat. No. 13-990-86) 193 Hand tally counter (Cat. No. 07905-6) 194 Hemocytometer, cell counter (Cat. No. 02-671-5) 195 Light microscope, inverted (Cat. No. 12-561-INV) Light microscope, upright (Cat. No. 12-561-3M) 196

197 Liquid nitrogen flask (Cat. No. 11-675-92) 198 Micropipetter, repeating (Cat. No. 21-380-9) 199 Pipetters, air displacement, single channel (0.5 –10µl (Cat. No. 21-377-191), 2 – 200 20 μl (Cat. No. 21-377-287), 20 – 200 μl (Cat. No. 21-377-298), 200 - 1000 μl 201 (Cat. No. 21-377-195)) 202 Refrigerator/freezer (Cat. No. 13-986-106A) 203 Shaker for 96-well plates (Cat. No. 14-271-9) 204 Sodium hydroxide (Cat. No. 5318-500) 205 Sonicating water bath (Cat. No. 15-335-30) 206 Tissue culture incubator with CO₂ and temperature control (Cat. No. 11-689-4) 207 Vacuum pump with liquid trap (side arm Erlenmeyer) (Cat. No. 01-092-29) 208 Vortex mixer (Cat. No. 12-814) 209 Equipment should be maintained and calibrated as per GLP guidelines and individual laboratory 210 SOPs. 211 7.3 Reference Standard, Controls, and Tissue Culture Supplies 212 All tissue culture reagents must be labeled to indicate source, identity, storage conditions and 213 expiration dates. Tissue culture solutions must be labeled to indicate concentration, stability 214 (where known), and preparation and expiration dates. 215 Equivalent tissue culture media and sera from another commercial source can be used, but must first be tested as described in **Section 17.0** to determine suitability for use in this test method. 216 217 The following are the necessary tissue culture reagents and possible sources based on their use in 218 the pre-validation studies: 219 17 β-estradiol (CAS RN: 50-28-2) [Sigma-Aldrich, Cat. No. E8875] 220 CellTiter-Glo[®] Luminescent Cell Viability Assay [Promega Cat. No. G7572] 221 Cryovial, 2 mL (Corning Costar) [Fisher Scientific Cat. No. 03-374-21] 222 Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 10009186R38] 223 Culture tube, 50 mL conical (Corning Costar) [Fisher Scientific Cat. No. 05-224 526C] 225 DMSO, U.S.P. analytical grade. [Sigma-Aldrich, Cat. No. 34869-100ML] 226 Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L 227 glucose, with sodium pyruvate, without phenol red or L-glutamine 228 [Mediatech/Cellgro, Cat. No. 17-205-CV]

229 Fetal Bovine Serum [Mediatech/Cellgro Cat. No. MT 35-010-CV] 230 Fetal Bovine Serum, charcoal/dextran treated, triple 0.1 µm sterile filtered 231 [Hyclone, Cat. No. SH30068.03] 232 Flavone (CASRN: 525-82-6) [Sigma-Aldrich, Cat. No. F2003] 233 Gentamycin Sulfate (G418), 50 mg/mL [Mediatech/Cellgro Cat. No. 30-234-CR] 234 L-glutamine, 29.2 mg/mL [Cellgro, Cat. No. 25005-CI] 235 Luciferase Assay System (10-Pack) [Promega Cat. No. E1501] 236 Lysis Solution 5X [Promega, Cat. No. E1531] 237 Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 µg/mL streptomycin 238 [Cellgro, Cat. No. 30-001-CI]. 239 Phosphate buffered saline (PBS, 1X) without calcium and magnesium [Cellgro, 240 Cat. No. 21-040-CV] Pipettes, serological: 2.0 mL [Sigma-Aldrich, Cat. No. P1736], 5.0 mL [Sigma-241 242 Aldrich, Cat. No. P1986], 25 mL [Sigma-Aldrich, Cat. No. P2486] Raloxifene (CASRN 84449-90-1) [Sigma-Aldrich Cat. No. R1402] 243 244 RPMI 1640 medium, containing L-glutamine [Mediatech, Cat. No. 10-040-CV] 245 Tissue culture flasks (Corning-Costar): 25 cm² (T25) [Fisher Cat. No. 10-126-28]; 75 cm² (T75) [Fisher Cat. No. 10-126-37]; and 150 cm² (T150) [Fisher Cat. No. 246 247 10-126-34] 248 Tissue culture plates (Corning-Costar): 96-well [Thomas Scientific Cat. No. 249 6916A05] 250 Trypsin (10X), 2.5% in Hank's balanced salt solution (HBSS), without calcium 251 and magnesium, without phenol red [Cellgro, Cat. No. 25-054-CI]. 252 All reagent lot numbers and expiration dates must be recorded in the study notebook. 253 8.0 PREPARATION OF TISSUE CULTURE MEDIA AND SOLUTIONS 254 All tissue culture media and media supplements must be quality tested before use in experiments 255 (see **Section 15.0**). 256 8.1 RPMI 1640 Growth Medium (RPMI) 257 RPMI 1640 is supplemented with 0.9% Pen-Strep and 8.0% FBS to make RPMI growth medium 258 (RPMI). 259 Procedure for one 549 mL bottle:

- 1. Remove FBS from -70°C freezer, and Pen-Strep from -20°C freezer and allow to equilibrate to room temperature.
- 262 2. Add 44 mL of FBS and 5 mL Pen-Strep to the bottle of RPMI 1640.
- 263 3. Label RPMI bottle as indicated in **Section 7.3**
- 264 Store at 2-8°C for no longer than six months or until the shortest expiration date of any media
- 265 component.

266 **8.2** Estrogen-Free DMEM Medium

- DMEM is supplemented to contain 4.5% charcoal/dextran treated FBS, 1.9% L-glutamine, 0.9%
- Pen-Strep.
- 269 Procedure for one 539 mL bottle:
- 1. Remove charcoal/dextran treated FBS from -70°C freezer, and L-glutamine and Pen-Strep from -20°C freezer and allow to equilibrate to room temperature.
- 272 2. Add 24 mL of charcoal/dextran treated FBS, 10 mL L-glutamine, and 5 mL Pen-273 2. Strep to one 500 mL bottle of DMEM.
- 274 3. Label estrogen-free DMEM bottle as indicated in **Section 7.3**
- 275 Store at 2-8°C for no longer than six months or until the shortest expiration date of any media
- 276 component.

277 8.3 1X Trypsin Solution

- 278 1X trypsin solution is prepared by dilution from a 10X premixed stock solution. The 10X stock
- solution should be stored in 10 mL aliquots in a -20°C freezer.
- 280 Procedure for making 100 mL of 1X trypsin:
- 1. Remove a 10mL aliquot of 10X trypsin from -20°C freezer and allow to equilibrate to room temperature.
- 283 2. Aliquot 1 mL trypsin (10X) along with 9 mL of 1X PBS into ten 15 mL centrifuge tubes.
- 285 3. Label 1X trypsin aliquots as indicated in Section 7.3
- 286 *IX trypsin should be stored at -20°C*.

287 8.4 1X Lysis Solution

- Lysis solution is prepared by dilution from a 5X premixed stock solution. Both the 5X and 1X
- solutions can be repeatedly freeze-thawed.

290 The procedure for making 10 mL of 1X lysis solution: 291 Thaw the 5X Promega Lysis solution and allow it to reach room temperature. 292 2. Remove 2 mL of 5X solution and place it in a 15 mL conical centrifuge tube. 293 3. Add 8 mL of distilled, de-ionized water to the conical tube. 294 4. Cap and shake gently until solutions are mixed. 295 Store at -20°C for no longer than 1 year from receipt. 296 8.5 **Reconstituted Luciferase Reagent** 297 Luciferase reagent consists of two components, luciferase buffer and lyophilized luciferase 298 substrate. 299 For long-term storage, unopened containers of the luciferase buffer and lyophilized luciferase substrate can be stored at -70°C for up to six months. 300 301 To reconstitute luciferase reagent: 1. Remove luciferase buffer and luciferase substrate from -70°C freezer and allow 302 303 them to equilibrate to room temperature. 304 2. Add 10 mL of luciferase buffer solution to luciferase substrate container and swirl 305 or vortex to mix, the Luciferase substrate should readily go into solution. 306 3. Luciferase substrate should readily go into solution. 307 4. After solutions are mixed aliquot to a 15mL centrifuge tube. 308 5. Store complete solution at -20° C. 309 Reconstituted luciferase reagent is stable for 1 month at -20°C. Reconstituted CellTiter-Glo® Reagent 310 8.6 CellTiter-Glo® reagent consists of two components, CellTiter-Glo® buffer and lyophilized 311 CellTiter-Glo® substrate. 312 For long term storage, unopened containers of the CellTiter-Glo® buffer and lyophilized 313 CellTiter-Glo® substrate can be stored at -70°C for up to one year. 314 To reconstitute CellTiter-Glo® reagent: 315 Remove CellTiter-Glo® buffer and CellTiter-Glo® substrate from -70°C freezer 316 and allow them to equilibrate to room temperature. 317 2. Add CellTiter-Glo® buffer solution to CellTiter-Glo® substrate container and 318 swirl or vortex gently to mix; the CellTiter-Glo® substrate should readily go into 319

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solution.

221		2 G 11T' G1 ® 1 + + 1 11 1'1 ' + 1 1'
321		3. CellTiter-Glo® substrate should readily go into solution.
322		4. After solutions are mixed aliquot to a 15mL centrifuge tube.
323		5. Store complete solution at –20°C.
324	Reconstit	uted CellTiter-Glo® reagent is stable for up to 3 months at –20°C.
325	9.0	OVERVIEW OF PROPAGATION AND EXPERIMENTAL PLATING OF
326		BG1Luc4E2 CELLS
327 328 329 330 331	grown as 1°C, 90% during wo	Luc4E2 (BG-1) cells are stored in liquid nitrogen in 2 mL cryovials. BG-1 cells are a monolayer in tissue culture flasks in a dedicated tissue culture incubator at $37^{\circ}\text{C} \pm 5\%$ humidity, and $5.0\% \pm 1\%$ CO ₂ /air. The cells should be examined on a daily basis orking days under an inverted phase contrast microscope, and any changes in gy and adhesive properties must be noted in the study notebook.
332333		I flasks containing cells at 80% to 90% confluence will usually yield a sufficient feells to fill four 96-well plates for use in experiments.
334	9.1	Procedures for Thawing Cells and Establishing Tissue Cultures
335 336		tissue culture media and solutions to room temperature by placing them under the ture hood several hours before use.
337 338		culture media, media supplements, and tissue culture plasticware must be quality ore use in experiments (Section 17.0).
339	9.1.1	Thawing Cells
340		1. Remove a cryovial of frozen BG-1 cells from the liquid nitrogen flask.
341 342		2. Facilitate rapid thawing by loosening the top slightly (do not remove top) to release trapped gasses and retightening it. Roll vial between palms.
343		3. Use a micropipette to transfer cells to a 50 mL conical centrifuge tube.
344		4. Rinse cryovial twice with 1X PBS and add PBS rinse material to the conical tube.
345		5. Add 20 mL of RPMI to the conical tube.
346347		6. Centrifuge at 1000 x g for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
348 349		7. Aspirate media from pellet and re-suspend it in 5 mL RPMI, drawing the pellet repeatedly through a 1.0 mL serological pipette to break up any clumps of cells.
350 351		8. Transfer cells to a T25 flask, place them in an incubator (see conditions in Section 9.0) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
352	9.1.2	Establishing Tissue Cultures

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- 353 Once cells have reached 80% to 90% confluence, transfer the cells to a T75 flask by performing, 354 for example, the following steps: 355 1. Remove the T25 flask from the incubator. 356 2. Aspirate the RPMI, then add 5 mL 1X PBS, making sure that the cells are coated 357 with PBS. 358 3. Aspirate 1X PBS, then add 1 to 2 mL 1X trypsin to the T25 flask, gently swirling 359 the flask to coat all cells with the trypsin. 360 4. Place the flask in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
 - 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.6. Confirm call detachment by examination under an inverted microscope. If calls
 - 6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, then hit the flask again.
 - 7. After cells have detached, add 5 mL PBS, and transfer the suspended cells to a 50 mL centrifuge tube. Wash the flask one additional time with 5 mL PBS.
 - 8. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular digestion by residual trypsin.
 - 9. Pellet the cells by centrifugation, as described in **Section 9.1.1**, and re-suspend the cells in 10 mL RPMI medium.
 - 10. Draw the pellet repeatedly through a 25 mL serological pipette to break up clumps of cells
 - 11. Transfer cells to a T75 flask, then place the flask in an incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
 - When cells have reached 80% to 90% confluence, transfer them into a T150 flask by performing, for example, the following steps:
 - 12. Remove the T75 flask from the incubator, aspirate the old media and add 5 mL 1X PBS.
 - 13. Aspirate 1X PBS, add 2 mL of 1X trypsin to the flask, and place it in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
 - 14. Repeat **steps 5** through **11** in **Section 9.1.2**, re-suspending the pellet in 20 mL of RPMI.
 - 15. Transfer cells to a T150 flask and place it in the incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
 - 16. Remove the T150 flask from the incubator.

38/	1 /	. Aspirate the RPMI and add 5 mL 1X PBS.
388	18	. Aspirate 1X PBS and add 3 mL 1X trypsin to the T150 flask, making sure that the
389		cells are coated with the trypsin.
390	19	. Incubate cells in an incubator (see conditions in Section 9.0) for 5 to 10 min.
391	20	. Detach cells by hitting the side of the flask sharply against the palm or heel of the
392		hand.
393394	21	. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes,
395		then hit the flask again.
396 397	22	. After cells have detached, add 5mL 1X PBS and transfer the suspended cells from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the flask,
398		then transfer to the 50 mL conical tube.
399 400	23	. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular digestion by residual trypsin.
401 402	24	Centrifuge at 1000 x g for eight minutes. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
403 404 405	25	Aspirate the media from the pellet and re-suspend it in 40 mL RPMI, drawing the pellet repeatedly through a 25 mL serological pipette to break up any clumps of cells.
406 407 408	26	. Transfer 20 mL of cell suspension to each of two T150 flasks, place them in an incubator (see conditions in Section 9.0) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
409 410		ngoing Tissue Culture Maintenance, Conditioning in Estrogen-free Medium, d Plating Cells for Experimentation
411 412 413	environment	g procedure is used to condition the BG1Luc4E2 cells to an estrogen-free prior to plating the cells in 96-well plates for analysis of estrogen dependent luciferase activity.
414 415 416 417 418	flasks into fo will use the l	issue culture maintenance and estrogen-free conditioning, split the two T150 culture our T150 flasks. Two of these flasks will be used for continuing tissue culture and RPMI media mentioned above. The other two flasks will be cultured in estrogen-free experimental use. Extra care must be taken to avoid contaminating the estrogen-free PMI.
419	1.	Remove both T150 flasks from the incubator.
420	2.	Aspirate the medium and rinse the cells with 5 mL 1X PBS.
120	2.	rispirate the inequality and thise the cens with 5 mil 17. 1 bb.

- DO NOT CITE, QUOTE, OR DISTRIBUTE 421 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask 422 to coat all cells with the trypsin. 423 4. Incubate cells in the incubator (see conditions in **Section 9.0**) for 5 to 10 min. 424 5. Detach cells by hitting the side of the flask sharply against the palm or heel of the 425 hand. 426 6. Confirm cell detachment by examination under an inverted microscope. If cells 427 have not detached, return the flask to the incubator for an additional 2 minutes, 428 then hit the flask again. 429 7. After cells have detached, add 5 mL 1X PBS to the first T150 flask and transfer 430 the suspended cells to the second T150 flask. 431 8. Transfer the contents of both flasks to a 50 mL conical tube. Repeat step 7 with an 432 additional 5 mL 1X PBS and transfer to the 50 mL conical tube. 433 9. Immediately add 20 mL estrogen-free DMEM to the 50 mL conical tube to inhibit 434 further cellular digestion by residual trypsin. 435 10. Centrifuge at 1000 x g for eight minutes. If a pellet of cells has not formed, 436 centrifuge for an additional 5 minutes. 437 11. Aspirate media from pellet and re-suspend it in 4 mL estrogen-free DMEM, 438 drawing the pellet repeatedly through a 1 mL serological pipette to break up 439 clumps of cells. At this point, cells are ready to be divided into the ongoing tissue culture and estrogen-free 440 441 conditioning groups. 442 921 Ongoing Tissue Culture Maintenance 443 1. Add 20 mL RPMI to two T150 flasks. 444 2. Add 220 µL G418 to the RPMI in the T150 flasks 445 3. Add 1 mL of cell suspension from **Section 9.2 step 11** to each flask. 446 4. Place T150 flasks in tissue culture incubator (see conditions in **Section 9.0**) and 447 grow to 80% to 90% confluence (approximately 48 to 72 hrs). 448 Tissue culture medium may need to be changed 24 hours after addition of G418 to 449 remove cells that have died because they do not express reporter plasmid. 450 6. G418 does not need to be added to the flasks a second time. 451 Repeat Section 9.2 steps 1-11 for ongoing tissue culture maintenance.
- 452 9.2.2 Conditioning in Estrogen-free Medium

1. Add 20 mL estrogen-free DMEM to two T150 flasks.

- 454 2. Add 150 µL G418 to the estrogen-free DMEM in the T150 flasks. 455 3. Add 1 mL of cell suspension from Section 9.2 step 11 to each flask. 456 4. Tissue culture medium may need to be changed 24 hours after addition of G418 to 457 remove cells that have died because they do not express reporter plasmid. 458 5. G418 does not need to be added to the flasks a second time. 459 6. Place the T150 flasks in the incubator (see conditions in **Section 9.0**) and grow to 460 80% to 90% confluence (approximately 48 to 72 hrs). 461 9.2.3 Plating Cells Grown in Estrogen-free DMEM for Experimentation 462 Remove the T150 flasks that have been conditioned in estrogen-free DMEM for 48 to 72 hours from the incubator. 463 464 2. Aspirate the medium, then rinse the cells with 5 mL 1X PBS. 465 3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask 466 to coat all cells with the trypsin. 467 4. Place the flasks in an incubator (see conditions in **Section 9.0**) for 5 to 10 min. 468 5. Detach cells by hitting the side of the flask sharply against the palm or the heel of 469 the hand. 470 6. Confirm cell detachment by examination under an inverted microscope. If cells 471 have not detached, return the flask to the incubator for 2 additional minutes, then 472 hit the flask again. 473 7. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells 474 from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the flask, then transfer to the 50 mL conical tube. 475 476 8. Immediately add 20 mL estrogen-free DMEM to each conical tube to inhibit 477 further cellular digestion by residual trypsin. 478 9. Centrifuge at 1000 x g for eight minutes. If a pellet of cells has not formed, 479 centrifuge for an additional 5 minutes. 480 10. Aspirate off the media from the pellet and re-suspend it in 20 mL DMEM, 481 drawing the pellet repeatedly through a 25 mL serological pipette to break up any 482 clumps of cells. 483 11. Pipette 15 µL of the cell suspension into the "v" shaped slot on the
 - 12. Using 100x magnification, view the counting grid.

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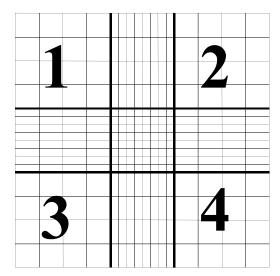
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hemocytometer grid, and allow cells to settle before counting.

hemocytometer. Ensure that the solution covers the entire surface area of the

13. The counting grid on the hemocytometer consists of nine sections, four of which are counted (upper left, upper right, lower left, and lower right, see **Figure 9-1**). Each section counted consists of four by four grids. Starting at the top left and moving clockwise, count all cells in each of the four by four grids. Some cells will be touching the outside borders of the square, but only count those that touch the top and right borders of the square. This value is then used in the calculation below to get to the desired concentration of 200,000 cells/mL.

Figure 9-1 Hemocytometer Counting Grid.



The volume of each square is 10^{-4} mL, therefore: Cells/mL = (average number per grid) x 10^{-4} mL. x 1/(starting dilution). Starting dilution: 20mL (for T150 flasks)

Harvested cells for a T150 flask are suspended in 20 mL of estrogen-free DMEM and sampled for determination of concentration of cells/mL.

Example Calculation:

- Grids 1, 2, 3, and 4 are counted and provide the following data:
 - o 50, 51, 49, and 50: average number of cells per grid is equal to 50.
- Cells/mL = 50 cells per grid \div 10⁻⁴ mL volume of grid = 50 X 10⁻⁴ cells/mL (or 500,000
- 505 cells/mL)

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- Total # of Cells Harvested = 500,000 cells/mL x 20 mL
- Desired Concentration (or Concentration Final) = 200,000 cells/mL
- Formula: (Concentration Final x Volume Final = Concentration Initial x Volume Initial)
- 510 Concentration Final = 200,000 cells/mL

511	Concentr	ation	$I_{\text{Initial}} = 500,000 \text{ cells/mL}$
512	Volume 1	nitial =	= 20 mL
513	Volume 1	Final —	to be solved for.
514	Therefore	e: 20	$00,000 \text{ cells/mL } \text{x Volume}_{\text{Final}} = 500,000 \text{ cells/mL } \text{x } 20 \text{ mL}$
515	Solving f	or V	olume $_{\text{Final}}$ we find = 50 mL
516 517		-	d 30 mL of estrogen-free DMEM to the cell suspension for a total volume of 50 ll yield the desired concentration of 200,000 cells/mL for plating.
518519520521			This dilution scheme will give a concentration of 200,000 cells/mL. 200 µL of this cell suspension is used for each well of a 96-well plate (i.e., 40,000 cells per well). Remove a 96-well plate from its sterile packaging. Use a repeater pipetter to
522			pipette:
523			• 200 μL of cell suspension into each well for range finder testing
524			• $200 \mu\text{L}$ of cell suspension into each well except the outside ring of
525			wells for comprehensive testing and add 200 μL of estrogen-free
526			DMEM to the outside ring of wells
527 528		16.	Incubate plate(s) in an incubator (see conditions in Section 9.0) for a minimum of 24 hours, but no longer than 48 hours before dosing.
529 530			sks containing cells at 80% to 90% confluence will typically yield sufficient cells well plates (not including the perimeter wells).
531	10.0	PR	EPARATION OF TEST SUBSTANCES
532533534535536	allowed t solutions subseque	o equ (exc nt tes	sed for dissolution of test substances is 100% DMSO. All test substances should be aillibrate to room temperature before being dissolved and diluted. Test substance ept for reference standards and controls) should not be prepared in bulk for use in sts. Test substances are to be used within 24 hours of preparation. Solutions should reable precipitate or cloudiness.
537 538			on on weighing, solubility testing, and calculation of final concentrations for test ference standards and controls is to be recorded in the study notebook.
539	10.1	Det	termination of Test Substance Solubility
540		1.	Prepare a 200 mg/mL solution of the test substance in 100% DMSO in a 4 mL
541			conical tube.
542		2.	Vortex to mix.

543 3. If the test substance does not dissolve at 200 mg/mL, prepare a 20 mg/mL solution and vortex as above. 544 545 4. If the test substance does not dissolve at 20 mg/mL solution, prepare a 2 mg/mL 546 solution in a 4 mL conical tube and vortex as above. 547 5. If the test substance does not dissolve at 2 mg/mL, prepare a 0.2 mg/mL solution 548 in a 4 mL conical tube and vortex as above. 549 6. Continue testing, using 1/10 less substance in each subsequent attempt until test 550 substance is solubilized in DMSO. 551 Once a solution of test substance has been obtained that does not have any visible precipitate or cloudiness in 100% DMSO, the solubility of the test substance must be determined in the 1% 552 553 DMSO/99% estrogen-free DMEM mixture used for LUMI-CELL® ER testing. 554 7. Add 2 µL of the highest concentration of the test substance/DMSO solution to a 555 13 mm test tube. 556 8. Add 400 µL estrogen-free DMEM to the test tube and vortex gently, 557 9. If cloudiness or precipitate develop, vortex for up to 10 minutes. 558 10. If vortexing does not dissolve test substance, sonicate test substance for up to 10 559 minutes. 560 11. If test substance has visible precipitate or is cloudy return to Section 10.1 step 7 561 to try the next lower concentration for the test substance. 562 The Testing Facility shall forward the results from the solubility tests assay to the SMT through 563 the designated contacts in electronic format and hard copy upon completion of testing. 564 11.0 PREPARATION OF REFERENCE STANDARD, CONTROL AND TEST 565 SUBSTANCE STOCK SOLUTIONS FOR RANGE FINDER AND COMPREHENSIVE TESTING 566 567 All information on preparation of test substances, reference standards and controls is to be 568 recorded in the study notebook. 569 11.1 Preparation of Ral/E2 Stock Solutions 570 E2 and raloxifene stocks are prepared separately and then combined into Ral/E2 stocks, which 571 are then used to prepare dosing solutions in **Section 12**. 572 11.1.1 E2 Stock Solution The final concentration of the E2 stock solution is 5.0 x 10⁻³ µg/mL. Prepare the E2 stock as 573 574 shown in **Table 11-1**.

575 Table 11-1 Preparation of E2 Stock Solution

Step #	Action	DMSO	E2 Concentration
1	Make a 10 mg/mL stock solution in 100% DMSO in a 4mL vial.	-	10 mg/mL
2	Transfer 10 µL E2 solution from Step #1 to a new 4 mL vial.	Add 990 μL of 100% DMSO. Vortex to mix.	100 μg/mL
3	Transfer 10 µL E2 solution from Step #2 to a new 4mL vial.	Add 990 μL of 100% DMSO. Vortex to mix.	1 μg/mL
4	Transfer 100 µL E2 solution from Step #3 to a new glass container large enough to hold 15 mL.	Add 9.90 mL of 100% DMSO. Vortex to mix.	1.0 x 10 ⁻² μg/mL
5	Transfer 5 mL E2 solution from Step #4 to a new glass container large enough to hold 15 mL	Add 5 mL of 100% DMSO. Vortex to mix.	5.0 x 10 ⁻³ μg/mL

576 11.1.2 Raloxifene Working Stock Solution

577 Prepare a 2.5 μg/mL raloxifene working stock solution as shown in **Table 11-2**.

578 Table 11-2 Preparation of Raloxifene Working Stock Solution

Step #	Action	DMSO	Raloxifene Concentration	
1	Make a 10 mg/mL solution of raloxifene in a 4 mL glass vial.	-	$1.0 \times 10^4 \mu g/mL$	
2	Transfer 10 µL raloxifene solution from	Add 990 μL of 100% DMSO.	100 μg/mL	
2	Step #1 to a new 4 mL vial.	Vortex to mix.		
2	Transfer 150 μL raloxifene solution from	Add 2.850 mL of 100% DMSO.	5 μg/mL	
3	Step #2 to a new 4 mL vial.	Vortex to mix.	5 μg/IIIL	
1	Transfer 1.5 mL raloxifene solution from	Add 1.5 mL of 100% DMSO.	2.5 μg/mL	
4	Step #3 to a new 13 mm test tube.	Vortex to mix.	2.5 μg/IIIL	

579 11.2 Ral/E2 Range Finder Stock

580 11.2.1 Raloxifene Dilutions

Number three 4 mL vials with the numbers 1 to 3 and use the raloxifene solution prepared in

Section 11.1.2 to make raloxifene dilutions as shown **Table 11-3**.

583 Table 11-3 Preparation of Raloxifene Reference Standard for Range Finder Testing

Step #	Action	DMSO	Raloxifene Concentration	
1	Transfer 500 μL of the raloxifene working	_	2.5 μg/mL	
1	stock solution to a new 4 mL vial.			
2	Transfer 50 μL of the raloxifene working	Add 500 μL of 100%	2.50 x 10 ⁻¹ μg/mL	
	stock solution to a new 4 mL vial.	DMSO. Vortex to mix.		
3	Transfer 2 µL of the raloxifene working stock	Add 500 μL of 100%	1.00 x 10 ⁻² μg/mL	
3	solution to a new 4 mL vial.	DMSO. Vortex to mix.	1.00 λ 10 μg/IIIL	

585 11.2.2 <u>Ral/E2 Dilutions for Range Finder Stock:</u>

Add 500 μ L of the 5 x 10⁻³ μ g/mL E2 solution prepared in **Section 11.1.1** to each of the

raloxifene dilution vials prepared in **Section 11.2.1**. Vortex each tube to mix. The final

concentrations for raloxifene and E2 are listed in **Table 11-4**.

Table 11-4 Concentrations of Raloxifene and E2 in the Ral/E2 Range Finder Stock
 Solution

Tube #	Raloxifene (µg/ml)	E2 (μg/ml)
1	1.25	2.5 x 10 ⁻³
2	1.25 x 10 ⁻¹	2.5 x 10 ⁻³
3	5.00 x 10 ⁻³	2.5 x 10 ⁻³

11.3 Ral/E2 Comprehensive Testing Stock

592 11.3.1 Raloxifene Dilutions for Comprehensive Testing Stock

Use the raloxifene solution prepared in **Section 11.1.2** to make a 9-point serial dilution of

raloxifene as shown **Table 11-5**.

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Table 11-5 Preparation of Raloxifene 9-Point Serial Dilution

Step #	Action	DMSO	Discard	Raloxifene Concentration
1	Transfer 500 μL of the raloxifene working stock solution to a new 4 mL vial.	-	1	2.5 μg/mL
2	Transfer 500 μL of the raloxifene working stock solution to a new 4 mL vial.	Add 500 μL of 100% DMSO. Vortex to mix.	-	1.25 μg/mL
3	Transfer 500 μL raloxifene solution from Step #2 to a new 4 mL vial.	Add 500 μL of 100% DMSO. Vortex to mix.	-	6.25 x 10 ⁻¹ μg/mL
4	Transfer 500 μL raloxifene solution from Step #3 to a new 4 mL vial.	Add 500 μL of 100% DMSO. Vortex to mix.	-	3.13 x 10 ⁻¹ μg/mL
5	Transfer 500 μL raloxifene solution from Step #4 to a new 4 mL vial.	Add 500 μL of 100% DMSO. Vortex to mix.	-	1.56 x 10 ⁻¹ μg/mL
6	Transfer 500 μL raloxifene solution from Step #5 to a new 4 mL vial.	Add 500 μL of 100% DMSO. Vortex to mix.	-	7.81 x 10 ⁻² μg/mL
7	Transfer 500 μL raloxifene solution from Step #6 to a new 4 mL vial.	Add 500 μL of 100% DMSO. Vortex to mix.	-	3.91 x 10 ⁻² μg/mL
8	Transfer 500 μL raloxifene solution from Step #7 to a new 4 mL vial.	Add 500 µL of 100% DMSO. Vortex to mix.		1.95 x 10 ⁻² μg/mL
9	Transfer 500 μL raloxifene solution from Step #8 to a new 4 mL vial.	Add 500 μL of 100% DMSO. Vortex to mix.	Discard 500 µL from Tube #9	9.77 x 10 ⁻³ μg/mL

- 597 11.3.2Ral/E2 Dilutions for Comprehensive Testing Stock:
- Add 500 μ L of the 5 x 10⁻³ μ g/mL E2 solution prepared in **Section 11.1.1** to each of the 9
- raloxifene dilution vials (including the working stock solution in Tube #1). Vortex each tube to
- 600 mix. The final concentrations for raloxifene and E2 are listed in **Table 11-6**.

Table 11-6 Concentrations of Raloxifene and E2 in the Ral/E2 Stock Solution

Tube #	Raloxifene (µg/mL)	E2 (µg/mL)
1	1.25	2.5 x 10 ⁻³
2	6.25 x 10 ⁻¹	2.5 x 10 ⁻³
3	3.13×10^{1}	2.5 x 10 ⁻³
4	1.56 x 10 ⁻¹	2.5 x 10 ⁻³
5	7.81×10^2	2.5 x 10 ⁻³
6	3.91 x 10 ⁻²	2.5 x 10 ⁻³
7	1.95 x 10 ⁻²	2.5 x 10 ⁻³
8	9.77 x 10 ⁻³	2.5 x 10 ⁻³
9	4.88 x 10 ⁻³	2.5 x 10 ⁻³

11.4 Flavone/E2 Stock Solution

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- To prepare the flavone/E2 stock solution, proceed as follows:
- 1. Prepare 1 mL of 10 mg/mL flavone
- Add 1 mL of the 5x10⁻³ μg/mL E2 (prepared as in Section 11.1.1) to the 10 mg/mL flavone. This will make a working solution of 5 mg/mL flavone with 2.5x10⁻³ μg/mL E2.

608 12.0 PREPARATION OF REFERENCE STANDARD, CONTROL AND TEST 609 SUBSTANCE DOSING SOLUTIONS FOR RANGE FINDER AND 610 COMPREHENSIVE TESTING

12.1 Preparation of Reference Standard and Control Dosing Solutions for Range Finder Testing

- Range finder testing is conducted on 96-well plates using three concentrations of Ral/E2 in
- duplicate as the reference standard. Three replicate wells for the DMSO, and E2 controls are
- 615 included on each plate.
- All "dosing solutions" of test substance concentrations are to be expressed as $\mu g/mL$ in the study
- notebook and in all laboratory reports.
- Dosing solutions are to be used within 24 hours of preparation.

619 12.1.1 Preparation of Ral/E2 Reference Standard Range Finder Dosing Solutions

- 1. Label two sets of 13 mm glass tubes with the numbers 1 to 3.
- Add 4 μL of Ral/E2 stock from tube #1 from Section 11.2.2 to the two 13 mm
 glass test tubes labeled #1.
 - 3. Add 4 µL of Ral/E2 stock from tube #2 from **Section 11.2.2** to the two 13 mm glass test tubes labeled #2. Repeat for tubes #3.
 - 4. Add 400 μL of estrogen-free DMEM to each tube and vortex to mix.

626 12.1.2 Preparation of DMSO Control Range Finder Dosing Solution

- 1. Add 4 μ L of 100% DMSO to three 13 mm glass test tubes.
- 628 2. Add 400 μL of estrogen-free DMEM to each tube and vortex to mix.

629 12.1.3 Preparation of E2 Control Range Finder Dosing Solution

- 1. Add 2 μL of the E2 stock from **Section 11.1.1** to three 13 mm glass test tube.
- 2. Add 400 μL of estrogen-free DMEM to each tube and vortex to mix.

632 12.2 Preparation of Test Substance Dosing Solutions for Range Finder Testing

- Range finder experiments are used to determine the concentrations of test substance to be used
- during comprehensive testing. Antagonist range finding for coded substances consists of six-
- 635 point, logarithmic serial dilutions.

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- To prepare test substance dosing solutions:
 - 1. Label two sets of six glass 13 mm test tubes with the numbers 1 through 6 and place them in a test tube rack. Perform a serial dilution of test substance as shown in **Table 12-1**.

Table 12-1 Preparation of Test Substance Serial Dilution for Range Finder Testing

Tube #	100% DMSO	Test Substance ¹	Final Volume
1	10 μL of test substance solution from Section 10.1		100 μL
2	90 μL	10 μL of test substance solution from Section 10.1	100 μL
3	90 μL	10 μL from Tube #2	100 μL
4	90 μL	10 μL from Tube #3	100 μL
5	90 μL	10 μL from Tube #4	100 μL
6	90 μL 10 μL from Tube #5		100 μL

Vortex tubes #2 through 5 before removing test substance/DMSO solution to place in the next tube in the series.

2. Transfer test substance/DMSO solutions to new tubes and add E2 as shown in **Table 12-2**.

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Table 12-2 Addition of E2 to Test Substance Serial Dilution for Range Finder Testing

Tube Number	Test Substance	E2	Remove	Estrogen- free DMEM ¹	Final Volume
1	Transfer 4 μL of test substance from Tube #1 in Section 12.2 step 1 to a new tube	Add 4 μL of the 5 x 10 ⁻³ μg/mL E2 solution prepared in Section 11.1.1 . Vortex to mix.	Remove and discard 4 µL from Tube #1	400 μL	404 μL
2	Transfer 4 µL of test substance from Tube #2 to a new tube	Add 4 μL of the 5 x 10 ⁻³ μg/mL E2 solution prepared in Section 11.1.1 Vortex to mix.	Remove and discard 4 µL from Tube #2	400 μL	404 μL
3	Transfer 4 µL of test substance from Tube #3 to a new tube	Add 4 μL of the 5 x 10 ⁻³ μg/mL E2 solution prepared in Section 11.1.1 . Vortex to mix.	Remove and discard 4 µL from Tube #3	400 μL	404 μL
4	Transfer 4 µL of test substance from Tube #4 to a new tube	Add 4 μL of the 5 x 10 ⁻³ μg/mL E2 solution prepared in Section 11.1.1 . Vortex to mix.	Remove 4 µL from Tube #4	400 μL	404 μL
5	Transfer 4 µL of test substance from Tube #5 to a new tube	Add 4 μL of the 5 x 10 ⁻³ μg/mL E2 solution prepared in Section 11.1.1 . Vortex to mix.	Remove 4 µL from Tube #5	400 μL	404 μL
6	Transfer 4 µL of test substance from Tube #6 to a new tube	Add 4 μL of the 5 x 10 ⁻³ μg/mL E2 solution prepared in Section 11.1.1 . Vortex to mix.	Remove 4 µL from Tube #6	400 μL	404 μL

¹Add 400 μL DMEM to each test tube after removing and discarding 4 μL of test substance/E2 mixture

Determination of whether a substance is positive in range finder testing and selection of starting concentrations for comprehensive testing will be discussed in **Section 14.0**.

12.3 Preparation of Reference Standard and Control Dosing Solutions for Comprehensive Testing

- Comprehensive testing is conducted on 96-well plates using nine concentrations of Ral/E2 in duplicate as the reference standard. Three replicate wells for the DMSO, E2 and flavone/E2 controls are included on each plate.
- All "dosing solutions" of test substance concentrations are to be expressed as $\mu g/mL$ in the study notebook and in all laboratory reports.
- Store dosing solutions at room temperature. Use within 24 hours of preparation.

658 12.3.1 <u>Preparation of Ral/E2 Reference Standard Dosing Solutions for Comprehensive</u> 659 <u>Testing</u>

In preparation for making Ral/E2 double serial dilutions, label two sets of nine glass 13 mm test

tubes with the numbers 1 through 9 and place them in a test tube rack. Tube number 1 will

contain the highest concentration of raloxifene (**Table 12-3**).

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Table 12-3 Preparation of Ral/E2 Reference Standard Dosing Solution

for Comprehensive Testing

Tube Number	Ral/E2 Stock	Estrogen- free DMEM	Final Volume
1	4 μL of Tube #1 from Section 11.3.2	400 μL	404 μL
2	4 μL of Tube #2 from Section 11.3.2	400 μL	404 μL
3	4 μL of Tube #3 from Section 11.3.2	400 μL	404 μL
4	4 μL of Tube #4 from Section 11.3.2	400 μL	404 μL
5	4 μL of Tube #5 from Section 11.3.2	400 μL	404 μL
6	4 μL of Tube #6 from Section 11.3.2	400 μL	404 μL
7	4 μL of Tube #7 from Section 11.3.2	400 μL	404 μL
8	4 μL of Tube #8 from Section 11.3.2	400 μL	404 μL
9	4 μL of Tube #9 from Section 11.3.2	400 μL	404 μL

665 12.3.2 <u>Preparation of DMSO Control Comprehensive Testing Dosing Solution</u>

- 1. Add 4 μ L of 100% DMSO to three 13 mm glass test tubes.
- 2. Add 400 µL of estrogen-free DMEM to each tube and vortex to mix.

668 12.3.3 <u>Preparation of E2 Control Comprehensive Testing Dosing Solution</u>

- 1. Add 2 μL of the E2 stock from **Section 11.1.1** to three 13 mm glass test tube.
- 2. Add 400 μL of estrogen-free DMEM to each tube and vortex to mix.

671 12.3.4 Preparation of Flavone/E2 Control Comprehensive Dosing Solution

- 1. Add 4 μL of flavone/E2 from **Section 11.4** to three 13 mm glass test tubes.
- 2. Add 400 μL of estrogen-free DMEM to each tube and vortex to mix.

12.4 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

675 Comprehensive testing experiments are used to determine whether a substance possesses ER

antagonist activity in the LUMI-CELL® ER test method. Antagonist comprehensive testing for

coded substances consists of 11-point, double serial dilutions, with each concentration tested in triplicate wells of the 96-well plate.

To prepare test substance dosing solutions for comprehensive testing:

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- 1. Determine the concentration at which maximal antagonism occurs in the range finder experiment. Start an 11-point serial dilution curve at a 20-fold higher concentration than the concentration causing a maximal antagonist response (i.e., if the maximum antagonist response occurred at 0.01 mg/mL, start the serial dilution curve at 0.2 mg/mL).
- 2. If there is no observable antagonist response in the range finder experiment, start the serial dilution at the maximum soluble concentration.
- 3. Label three sets of 11 glass 13 mm test tubes with the numbers 1 through 11 and place them in a test tube rack. Prepare three serial dilutions as in **Table 12-4**.

Table 12-4 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

Tube Number	100% DMSO	Test Substance ¹	Discard	E2 Testing Stock	Discard	Estrogen- free DMEM ²	Final Volume
1	-	4 μL of test substance solution from Section 10.2.4 step 1	-	4 μL	4 μL	400 μL	404 μL
2	4 μL	4 μL of test substance solution from Section 10.2.4 step 1	-	4 μL	4 μL	400 μL	404 μL
3	4 μL	4 μL from Tube #2	-	4 μL	4 μL	400 μL	404 μL
4	4 μL	4 μL from Tube #3	-	4 μL	4 μL	400 μL	404 μL
5	4 μL	4 μL from Tube #4	-	4 μL	4 μL	400 μL	404 μL
6	4 μL	4 μL from Tube #5	-	4 μL	4 μL	400 μL	404 μL
7	4 μL	4 μL from Tube #6	-	4 μL	4 μL	400 μL	404 μL
8	4 μL	4 μL from Tube #7	-	4 μL	4 μL	400 μL	404 μL
9	4 μL	4 μL from Tube #8	-	4 μL	4 μL	400 μL	404 μL
10	4 μL	4 μL from Tube #9	-	4 μL	4 μL	400 μL	404 μL
11	4 μL	4 μL from Tube #10	4 μL	4 μL	4 μL	400 μL	404 μL

Vortex tubes #2 through 10 before removing test substance/DMSO solution to place in the next tube in the series.

²Vortex all tubes to mix media, test substance, and E2.

693 13.0 GENERAL PROCEDURES FOR THE TESTING OF CODED SUBSTANCES

- Range finder experiments are used to determine the concentrations of test substance to be used
- during comprehensive testing. Comprehensive testing experiments are used to determine whether
- a substance possesses ER antagonist activity in the LUMI-CELL® ER test method.
- 697 General procedures for range finder and comprehensive testing are nearly identical. For specific
- details (such as plate layout) of range finder testing see **Section 14.0**. For specific details of
- 699 comprehensive testing, see **Section 15.0**.

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700 13.1 Application of Reference Standard, Control and Test Substances

- 1. Remove the 96-well plates (from **Section 9.2.3 step 18)** from the incubator; inspect them using an inverted microscope. Only use plates in which the cells in all wells receive a score of 1 according to **Table 11-1**.
 - 2. Remove medium by inverting the plate onto blotter paper. Gently tap plate against the bench surface to remove residual liquid trapped in the wells.
 - 3. Add 200 µL of medium, reference standard, control or test substance to each well (see **Sections 14.0** and **15.0** for specific plate layouts).
 - 4. Return plates to incubator (see **Section 9.0** for details) for 19 to 24 hours to allow maximal induction of luciferase activity in the cells.

710 13.1.1 <u>Preparation of Excel® Data Analysis Template</u>

- 1. In Excel[®], open a new "AntICCVAMTemplate" and save it with the appropriate project name as indicated in the NICEATM Style Guide.
- 2. Add appropriate information regarding the assay to the "Compound Tracking" tab
- 3. Enter substance testing information to the "List" page (i.e. Project /Sample ID, Concentration, and Comments (or compound name). This should populate the "Template", "Compound Mixing" and "Visual Inspection" tabs with the appropriate information for the experiment.
- 4. Save the newly named project file.
- 5. Print out either the "List" or "Template" page for help with dosing the 96-well plate. Sign and date the print out and store in study notebook.

722 **13.2** Visual Evaluation of Cell Viability

1. 19 to 24 hours after dosing the plate, remove the plate from the incubator and remove the media from the wells by inverting the plate onto blotter paper. Gently tap plate against the bench surface to remove residual liquid trapped in the wells.

- Use a repeat pipetter to add 50 μL 1X PBS to all wells. Immediately remove PBS by inversion.
 - 3. Using an inverted microscope, inspect all of the wells used in the 96-well plate and record the visual observations using the scores in **Table 13-1**.

Table 13-1 Visual Observation Scoring

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Viability Score	Brief Description ¹	
1	Normal Cell Morphology and Cell Density	
2	Altered Cell Morphology and/or Small Gaps between Cells	
3	Altered Cell Morphology and/or Large Gaps between Cells	
4	Few (or no) Visible Cells	
1P	Score of 1 with Precipitate	
2P	Score of 2 with Precipitate	
3P	Score of 3 with Precipitate	
4P	Score of 4 with Precipitate	
5P	Unable to View Cells Due to Precipitate	

731 Reference micrographs will be provided by NICEATM.

13.3 Lysis of Cells for LUMI-CELL® ER

- 1. Apply the reflective white backing tape to the bottom of the 96-well plate (this will increase the effectiveness of the luminometer).
- 2. Add 30µL 1X lysis reagent to the assay wells and place the 96-well plate on an orbital shaker for one minute.
- 3. Remove plate from shaker and measure luminescence (as described in **Section 11.5**).

13.4 CellTiter-Glo® Assessment of Cell Viability

- When considered necessary, a quantitative evaluation of cell viability will be performed with the Promega CellTiter-Glo® assay system. CellTiter-Glo® uses luminescence as an indicator of the number of cells per plate and therefore must be conducted in parallel with the LUMI-CELL® ER test method (i.e., both test methods cannot be conducted on the same plate).
 - 1. Dose and incubate cells under the same conditions as for LUMI-CELL® ER.
 - 2. Remove plates from incubator and discard the medium by inverting the plate onto blotter paper. Gently tap plate against the bench surface to remove residual liquid trapped in the wells.

- Use a repeat pipetter to add 50 μL 1X PBS to all assay wells. Immediately remove PBS by inversion.
 - 4. Examine all wells used under an inverted microscope. Make notes of any well with codes described in **Table 13-1**.
 - 5. Place white backing tape on the bottom of the 96-well plate.
- 6. Add 100 μL estrogen-free DMEM to each well containing cells.
- 7. Add 100 μL CellTiter-Glo® reagent to each well containing cells.
- 755 8. Place plate on an orbital shaker for 1 minute to induce cell lysis.
- 756 9. Incubate (see **Section 9.0** for details) for 10 min.
- 757 10. Measure luminescence promptly as in **Section 13.5**. Do *not* add luciferase reagent to CellTiter-Glo[®] plates.

759 13.5 Measurement of Luminescence

- Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and
- with software that controls the injection volume and measurement interval. Light emission from
- each well is expressed as relative light units (RLU) per well. The luminometer output is saved as
- raw data in an Excel[®] spread sheet. A hard copy of the luminometer raw data should be signed,
- dated and stored in the study notebook.

765 **13.6 Data Analysis**

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- 766 LUMI-CELL® ER uses an Excel® spreadsheet to collect and adjust the RLU values obtained
- 767 from the luminometer and a GraphPad PRISM® template to analyze and graph data. Plate
- reduction is calculated using unadjusted RLU values.
- 769 The Excel® spreadsheet subtracts background luminescence (average DMSO solvent control
- RLU value) from test substance, reference standard and control RLU values. Test substance,
- reference standard, and control RLU values are then adjusted relative to the highest Ral/E2
- reference standard RLU value, which is set to 10,000. After adjustment, values are transferred to
- 773 GraphPad PRISM® for data analysis and graphing.
- 774 13.6.1 Correction and Adjustment of Luminometer Data
- 775 The following steps describe the procedures required to populate the Excel® spreadsheet that has
- been configured to collect and adjust the RLU values obtained from the luminometer.
- 777 1. Open the raw data file and the corresponding experimental Excel® spreadsheet
- 778 from **Section 13.1.1**.

779 2. Copy the raw data using the Excel® copy function, then paste the copied data into 780 cell C22 of the "RAW DATA" tab in the experimental Excel® spreadsheet using 781 the Paste Special – Values command. This position corresponds to position B2 in 782 the table labeled Table 1 in this tab. 3. Examine the DMSO data in Table 1 of the Excel® spreadsheet to determine 783 784 whether there are any potential outliers. See **Section 13.6.2** for further explanation 785 of outlier determinations. 786 4. If an outlier is identified, perform the following steps to remove the outlier from 787 calculations: 788 correct the equation used to calculate DMSO background in Table 1, 789 e.g., if outlier is located in cell G24, adjust the calculation in cell H42 to 790 read = AVERAGE(F24.H24,I24)791 then correct the equation used to calculate the average DMSO value in 792 Table 2, e.g., following the above example, adjust cell M44 to read 793 =AVERAGE(F36,H36,I36) 794 then correct the equation used to calculate the standard deviation of the 795 DMSO value in Table 2, e.g., following the above example, adjust cell 796 M45 to read =STDEV(F36,H36,I36) 5. Excel® will automatically subtract the background (the average DMSO control 797 798 value) from all of the RLU values in Table 1 and populate Table 2 with these 799 adjusted values. 800 6. To calculate plate reduction, identify the cell containing the Ral/E2 replicate that has the lowest RLU value and the cell containing the RLU value for the same 801 802 concentration in the corresponding Ral/E2 replicate (e.g., if the lowest RLU value 803 for Ral/E2 is located in cell E23, the corresponding cell would be E22). 804 7. Identify the cell containing the Ral/E2 replicate that has the highest RLU value and the cell containing the RLU value for the same concentration in the 805 806 corresponding Ral/E2 replicate 807 8. Click into cell D16 and enter the cell numbers from **Section 13.6.1 step 6** into the 808 numerator and the cell numbers from **step 7** into the denominator. 809 9. Click on the "ER Antagonist Report" tab.

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10. The data for the Ral/E2 reference standard, DMSO, E2, and Flavone/E2 replicates populate the left portion (columns A-F) of the spreadsheet. The data is automatically placed into an Excel® graph.

- 11. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell D2 of "ER Antagonist Report" tab and check the formula contained within that cell. The divisor should be the cell number of the cell containing the highest averaged Ral/E2 RLU value (column E).
- 12. Forecast the EC50 value using the formula (FORECAST(x,known_y's,known_x's), where x = the data point for which you wish to predict a value [i.e., the median RLU], known y's = the three RLU values that constitute the middle of the linear portion of the concentration-response curve, and known x's = the concentrations corresponding to the known y's).
- 13. Copy the data into GraphPad PRISM® for graphing and analysis as indicated in the NICEATM PRISM® users guide.

824 13.6.2 <u>Determination of Outliers</u>

- The Study Director will use good statistical judgment for determining "unusable" wells that will be excluded from the data analysis and will provide an explanation in the study notebook for any
- 827 excluded data. This judgment for data acceptance will include Q test analysis.
- The formula for the Q test is:
- 829 $\frac{Outlier Nearest \ Neighbor}{Range (Highest Lowest)}$

where the outlier is the value proposed for exclusion, the nearest neighbor is the value closest to the outlier, and the range is the range of the three values (Q values for samples sizes from 3 to 10 are provided in Table 13-2). For example, if the value of this ratio is greater than 0.94 (the Q value for the 90% confidence interval for a sample size of three) or 0.76 (the Q value for the 90% confidence interval for a sample size of four), the outlier may be excluded from data analysis.

Table 13-2 Q Test Values

Number Of Observations	Q Value
2	-
3	0.94
4	0.76
5	0.64
6	0.56
7	0.51
8	0.47
9	0.44
10	0.41

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- 837 For E2 reference standard replicates (sample size of two), any adjusted RLU value for a replicate 838 at a given concentration of E2 is considered and outlier if its value is more than 20% above or 839 below the adjusted RLU value for that concentration in the historical database. 840 13.6.3 Acceptance Criteria 841 Acceptance or rejection of a test is based on evaluation of reference standard and control results 842 from each experiment conducted on a 96-well plate. Results are compared to quality controls 843 (QC) for these parameters derived from the historical database, which are summarized below. 844 Reduction: Plate reduction, as measured by dividing the averaged highest Ral/E2 845 reference standard RLU value by the averaged lowest Ral/E2 reference standard 846 RLU value, must be greater than three fold. 847 Reference standard results: Calculated Ral/E2 reference standard IC₅₀ values must 848 be within 2.5 times the standard deviation of the historical database IC₅₀ mean 849 value. 850 Solvent control results: DMSO control RLU values must be within 2.5 times the 851 standard deviation of the historical solvent control mean RLU value. 852 E2 control results: E2 control RLU values must be within 2.5 times the standard 853 deviation of the historical E2 control mean RLU value. 854 Positive control results: Flavone/E2 control RLU values must be within 2.5 times 855 the standard deviation of the historical database flavone/E2 control mean RLU 856 value.

RANGE FINDER TESTING

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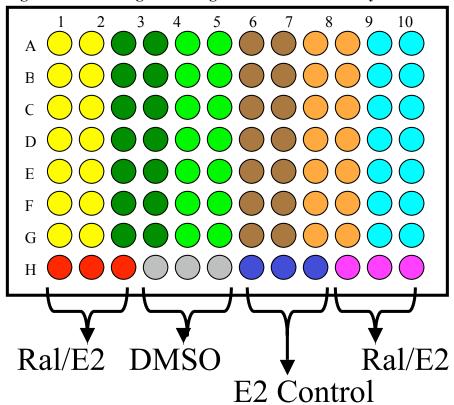
Antagonist range finding for coded substances consists of six-point, logarithmic serial dilutions tested in duplicate wells of the 96-well plate. **Figure 14-1** contains a template for the plate layout used in antagonist range finder testing.

An experiment that fails any single acceptance criterion will be discarded and repeated.

Figure 14-1 Antagonist Range Finder Test Plate Layout

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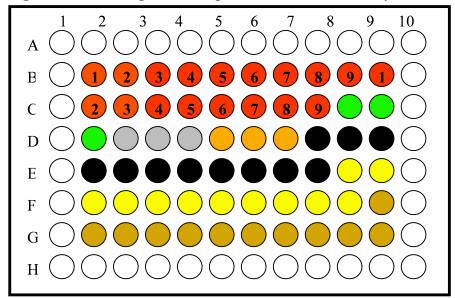
- Three Point Ral/E2 Reference Standard Replicate 1
- Three Point Ral/E2 Reference Standard Replicate 2
- DMSO Control (1% v/v)
- E2 Control (2.5 x 10⁻⁵ μg/mL)
- Range Finder for Sample #1
- Range Finder for Sample #2
- Range Finder for Sample #3
- Range Finder for Sample #4
- Range Finder for Sample #5
- Range Finder for Sample #6

- Evaluate whether range finder experiments have met the acceptance criteria (see **Section 13.6.3**) graph the data as described in the NICEATM PRISM® users guide.
- To determine starting concentrations for comprehensive testing use the following criteria:
 - If there are no points on the test substance concentration curve that are less than the mean value of the E2 control minus three times the standard deviation from that mean, the highest concentration used in comprehensive testing is the limit dose or the maximum soluble dose.
 - If there are points on the test substance concentration curve that are below the mean value of the E2 standard control minus three times the standard deviation from that mean, select a concentration that is a single log dilution higher than the concentration giving the lowest adjusted RLU value in the range finder and use that as the highest concentration for comprehensive testing.
 - If a substance exhibits a biphasic concentration curve, the range finder experiment should be repeated unless the proposed concentration range for the comprehensive studies will include all concentrations of the biphasic region in the range finding study. If the range finder experiment is repeated and the substance still exhibits a biphasic concentration curve, comprehensive testing must be conducted on the peak of the biphasic curve at the lowest test substance concentration. If the substance is negative at this lowest concentration, then test at the higher concentration. For either peak of the concentration curve, select a concentration that is a single log dilution higher than the concentration giving the lowest adjusted RLU value in the range finder and use that as the highest concentration for comprehensive testing.

15.0 COMPREHENSIVE TESTING

Antagonist comprehensive testing for coded substances consists of 11-point, double serial dilutions, with each concentration tested in triplicate wells of the 96-well plate. **Figure 15-1** contains a template for the plate layout to be used in antagonist comprehensive testing.

Figure 15-1 Antagonist Comprehensive Test Plate Layout



- 9 point Ral/E2 Reference Standard Dose Response Curve
- Flavone Control (25 μg/mL)
- DMSO Control (1%)

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- - E2 Control (2.5 x 10⁻⁵ μg /mL)
- Comprehensive Dose Response Sample #1, Replicate #1
- Comprehensive Dose Response Sample #1, Replicate #2
- Comprehensive Dose Response Sample #1, Replicate #3

Evaluate whether comprehensive experiments have met acceptance criteria (see **Section 13.6.3**) and graph the data as described in the NICEATM PRISM[®] users guide.

- If the substance has been tested up to the limit dose or the maximum soluble dose without causing a significant decrease in cell viability, and there are no points on the concentration curve that are below the line indicating the mean minus three times the standard deviation of the E2 control, the substance is considered negative for antagonism.
- If the substance has been tested up to the limit dose and there are points on the concentration curve that are below the line indicating the mean minus three times the standard deviation of the E2 control, but cell viability, as measured by

906 CellTiter-Glo[®] falls below 80%, or a visual inspection score of 2 or greater, at all 907 points falling below the E2 line, the substance is considered negative for antagonism. 908 909 If there are points on the test substance concentration curve that are below the line 910 indicating the mean minus three times the standard deviation of the E2 control 911 that do not cause a decrease in cell viability below 80% as measured by CellTiter-912 Glo[®], or a visual inspection score of 2 or greater, the substance is positive for 913 antagonism. 914 o Points in the test substance concentration curve that cause a decrease in 915 cell viability to below 80% as measured by CellTiter-Glo® or a visual 916 inspection score of 2 or greater, are not included in data analyses. 917 16.0 USE OF THE HISTORICAL DATABASE TO GENERATE QC CHARTS 918 The historical database is maintained in order to ensure that the test method is functioning properly. The historical database is maintained as an Excel® spreadsheet that is separate from the 919 920 spreadsheets used to report the data for individual experiments. The controls used to develop the 921 historical database are used as one of the criteria for determining a valid test. 922 Results collected during Phase I will be compared to historical control data established during the LUMI-CELL® ER Protocol Standardization Study. Reference standard and control data 923 924 collected during Phase I will be used to compile the initial historical database. Reference 925 standard and control data collected during Phase IIa will be added to the historical database 926 compiled in Phase I and this combined historical database will be used to establish acceptance 927 criteria for Phase II. Reference standard and control data collected during Phase IIb will be added 928 to the historical database compiled in Phases I and IIa and this combined historical database will 929 be used to establish acceptance criteria for Phases III and IV. **LUMI-CELL® ER Antagonist QC Charts** 930 16.1 1. Open the Excel® spreadsheet labeled LUMIAntagonistQC. 931 932 Save this sheet under a new name, adding the laboratory designator to the file name (e.g., for Laboratory A, the new name would be ALUMIAntagonistQC). 933 934 16.1.1 Flavone/E2 1. Open Excel® spreadsheet XDSLUMIAntQC. 935 936 2. Click on flavone tab and enter the date, plate number (name), and the average 1.56 x 10⁻³ µg/mL RLU value for ral/E2 (data located in column F on the "List" 937 938 tab of the antagonist report file).

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- 3. Enter the three values for flavone/E2 into column D.
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- 4. The mean and 2.5 times the standard deviation plus (and minus) the mean are calculated automatically.

5. Check the Scatter Charts tab to see if the average value for flavone/E2 falls within 2.5 times the standard deviation (e.g., **Figure 16-1**) of the historical mean. If the mean flavone/E2 value falls within the 2.5 times the standard deviation area, it passes QC. Otherwise it fails QC and the experiment must be repeated.

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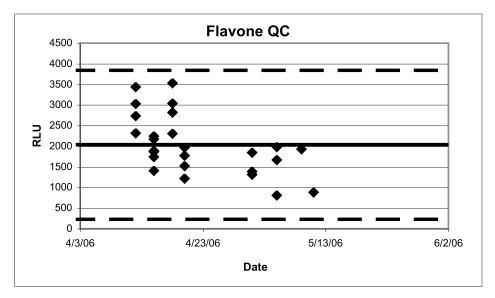
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Acceptance or rejection of the flavone/E2 control data is based on whether the data for a given experiment falls within 2.5 times the standard deviation from the historical mean RLU value.

Figure 16-1 Example Scatter Chart of the Flavone/E2 Control QC^{1,2,3}



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¹Each point represents a single experiment.

²The solid line represents the historical mean RLU value for the flavone control.

³The two dashed lines represent the historical mean RLU value for the flavone

control plus and minus 2.5 times the standard deviation from the historical mean.

16.1.2 <u>E2 Control</u>

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1. Click on the E2 control tab. The date, study name, and $1.56 \times 10^{-3} \,\mu\text{g/mL}$ ral/E2 data should be populated in the appropriate position.

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2. Enter the values for the E2 control into column D.

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3. The mean and 2.5 times the standard deviation plus (and minus) the mean are calculated automatically.

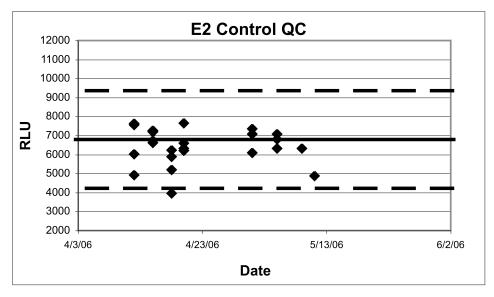
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4. Check the scatter charts tab to see if the average value for the E2 control falls within the 2.5 times the standard deviation (**Figure 16-2**) from the historical

mean. If the value falls within the 2.5 times the standard deviation area, the E2 control passes QC. Otherwise it fails QC and the study must be repeated.

Acceptance or rejection of the E2 control data is based on whether the data for a given experiment falls within 2.5 times the standard deviation from the historical mean RLU value.

Figure 16-2 Example Scatter Chart of the E2 Control QC^{1,2,3}



¹Each point represents a single experiment.

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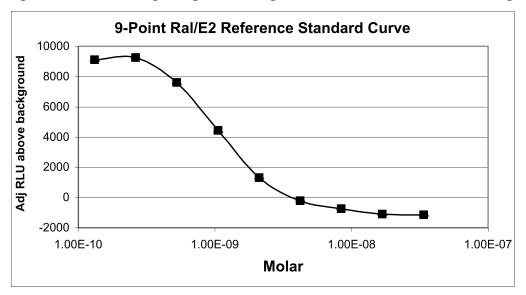
16.1.3 9-Point Ral/E2 Reference Standard QC

- 1. Enter the experiment date and name, and copy and paste the adjusted RLU values for Ral/E2 into the appropriate slots on the tab labeled Ral E2 Standard Curve.
- 2. The Ral/E2 standard curve is automatically graphed to ensure a normal sigmoidal shape (see **Figure 16-3** for an example curve).

²The solid line represents the historical mean RLU value for the E2 control.

³The two dashed lines represent the historical mean RLU value for the E2 control plus and minus 2.5 times the standard deviation from the historical mean.

977 Figure 16-3 Example Figure of a Sigmoidal Ral/E2 Concentration Response Curve¹



¹The points on the line represent the averaged Ral/E2 values for a single experiment.

16.1.4 <u>IC₅₀ Tracking Data</u>

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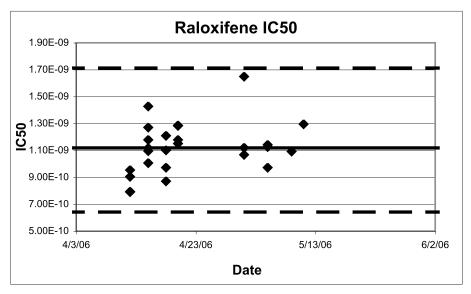
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- 1. Enter the date and plate ID into the first two columns of the IC₅₀ Tracking Data tab.
- 2. Link the EC_{50} data from the 9-point Ral/E2 Curve QC tab to the column to the right of the plate information.
- 3. Column E calculates the percent deviation from the historical database IC₅₀ value.
- 4. The mean and 2.5 times the standard deviation plus (and minus) the mean for the IC_{50} deviation are calculated automatically.
- 5. Check the Scatter Charts tab to see whether the experimental IC₅₀ value falls within the 2.5 times the standard deviation (**Figure 16-4**). If the value falls within the 2.5 times the standard deviation area, it passes QC. If the value does not fall within the 2.5 times the standard deviation, it fails QC and the experiment must be repeated.

Figure 16-4 Example Scatter Chart of the Ral/E2 IC₅₀ Control QC^{1,2,3}



995 ¹Each point represents a single experiment.

²The solid line represents the historical mean RLU value for the Ral/E2 EC₅₀.

 3 The two dashed lines represent the historical mean RLU value for the Ral/E2

control plus and minus 2.5 times the standard deviation from the historical mean.

Acceptance or rejection of Ral/E2 IC₅₀ data is based on whether the data falls within 2.5 times the standard deviation from the mean for the historical Ral/E2 IC₅₀ RLU value.

16.1.5 DMSO

- 1. The date and experiment name should populate automatically
- 2. Enter all of the DMSO values from Table 1 on the "Raw Data" tab on the Excel® spreadsheet which passed the outlier test, into the areas marked DMSO 1, DMSO 2, DMSO 3, and DMSO 4.
- 3. The average RLU value for DMSO is then calculated under the "mean" column.
- 4. The mean and 2.5 times the standard deviation plus (and minus) the mean for the DMSO deviation are calculated automatically.
- 5. Check the Scatter Charts tab to see whether the average value for DMSO falls within 2.5 times the standard deviation (**Figure 16-5**) from the mean. If the value falls within the 2.5 times the standard deviation area, the DMSO passes QC. If the value does not fall within the 2.5 times the standard deviation, it fails QC and the experiment must be repeated..

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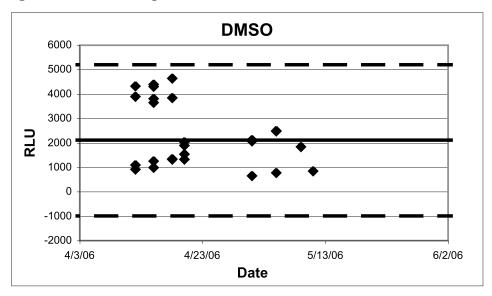
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Figure 16-5 Example Scatter Chart of the DMSO Control QC^{1,2,3}



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¹Each point represents a single experiment.

²The solid line represents the historical mean RLU value for the DMSO control.

³The two dashed lines represent the historical mean RLU value for the DMSO

control plus and minus 2.5 times the standard deviation from the historical mean.

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Acceptance or rejection of the DMSO control data is based on whether the data for a given experiment falls within 2.5 times the standard deviation from the historical mean RLU value.

1024 16.1.6 <u>Reduction</u>

1025 Reduction is calculated by dividing the highest RLU value for each of the Ral/E2 standards by

the lowest Ral/E2 RLU value. To calculate reduction, follow the steps below:

1027 Enter the reduction value from the "Raw Data" tab on the Excel® spreadsheet. If the value is

greater than or equal to 3, the experiment passed QC. An induction value of less than 3 fails

induction QC and the experiment must be repeated.

17.0 QUALITY TESTING OF MATERIALS

All information pertaining to the preparation and testing of media, media supplements, and other

materials should be recorded in the Study Notebook.

17.1 Tissue Culture Media

Each lot of tissue culture medium must be tested in a single growth flask of cells before use in

ongoing tissue culture or experimentation (**note:** each bottle within a given lot of

1036 Charcoal/Dextran treated FBS must be tested separately).

- 1. Every new lot of media (RPMI and DMEM) and media components (FBS, Charcoal/Dextran treated FBS, and L-glutamine) must first be tested on the LUMI-CELL® ER assay prior to being used in any GLP acceptable assays.
 - 2. Add 4 µL of DMSO (previously tested) into four separate 13 mm tubes.
 - 3. Add 400 µL media (to be tested) to 13 mm tube.
 - 4. Dose an experimental plate as in **Section 12.0**, treating the media being tested as a test substance.
 - 5. Analyze 96-well plate as described in **Section 12.0**, comparing the data from the DMSO controls made using previously tested tissue culture media to the new media being tested.
 - 6. Use the QC charts to determine if the new media with DMSO lies within 2.5 standard deviation of the mean for the media. If the RLU values for the new media with DMSO are within 2.5 standard deviation of the mean for the historical data on DMSO, the new lot of media is acceptable. If the RLU values for the new media with DMSO are not within 2.5 standard deviation of the mean for the historical data the new lot may not be used in the assay.
 - 7. Note date and lot number in study notebook.
 - 8. If the new bottle passes the QC as described in **Section 15.1 step 6**, apply the media to a single flask cells and observe the cells growth and morphology over the following 2 to 3 days. If there is no change in growth or morphology, the new media is acceptable for use.

17.2 G418

- 1. New lots of G418 must first be tested on the LUMI-CELL® ER assay prior to being used in any GLP acceptable assays.
- 2. Add 220 μL of G418 (previously tested) to a single flask containing cells growing in RPMI.
- 3. Add 220 μ L of G418 (to be tested) to a different flask containing cells growing in RPMI.
- 4. Observe cellular growth and morphology in both tissue culture flasks over a 48 to 72 hour period. If there are no differences in observed growth rate and morphology between the two flasks, the new G418 lot is acceptable.
- 5. If cellular growth is decreased, or the cells exhibit abnormal morphology, the new lot of G418 is not acceptable.
- 6. Note date and lot number in study book.

17.3 DMSO

- 1. Every new bottle of DMSO must be tested on the LUMI-CELL® ER assay prior to use in any GLP acceptable assays.
 - 2. Add 4 µL of DMSO (to be tested) into four separate 13 mm tubes.
 - 3. Add 400 µL media (previously tested) the same tubes.
 - 4. Dose an experimental plate as in **Section 15.0**, treating the media being tested as a test substance.
 - 5. Analyze 96-well plate as described in **Section 15.0**, comparing the data from the DMSO controls made using previously tested tissue culture media to the new media being tested.
 - 6. Use the QC charts to determine if the new media with DMSO lies within 2.5 standard deviation of the mean for the media. If the RLU values for the new media with DMSO are within 2.5 standard deviation of the mean for the historical data on DMSO, the new lot of media is acceptable. If the RLU values for the new media with DMSO are not within 2.5 standard deviation of the mean for the historical data the new lot may not be used in the assay.
 - 7. Use the QC charts to determine if the new DMSO is within 2.5 standard deviation of the mean for DMSO background. If the RLU for the new DMSO is within 2.5 standard deviation of the mean for the historical data on DMSO, then the new bottle of DMSO is acceptable; otherwise the new bottle may not be used in the assay.
 - 8. Note the date, lot number, and bottle number in study book.
 - 9. If no DMSO has been previously tested, test several bottles as described in **Section 15.3**, and determine whether any of the bottles of DMSO have a higher average RLU than the other bottle(s) tested. Use the DMSO with the lowest average RLU for official experiments.

17.4 Plastic Tissue Culture Materials

- 1. Grow one set of cells, plate them for experiments on plastic ware from the new lot and one set of cells in the plastic ware from a previous lot, and dose them with E2 reference standard and controls.
- 2. Perform the LUMI-CELL® ER experiment with both sets of cells.
- 3. If all of the analysis falls within acceptable QC criteria, then the new manufacturer's products may be used.

1104	18.0 REFERENCES
1105	ICCVAM. 2001. Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses
1106	for Acute Toxicity. NIH Pub. No. 01-4500. Research Triangle Park, NC: National Institute of
1107	Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/invidocs/
1108	guidance/iv_guide_ndf [accessed 31 August 2006]